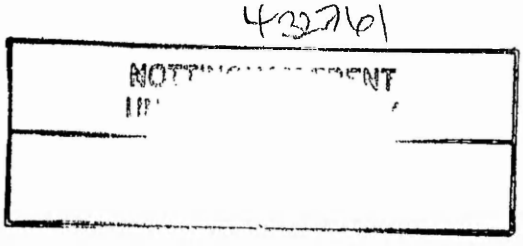
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The use of transglutaminase in the modification of collagen for the development of biomaterials

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A Thesis submitted in partial fulfillment of the requirements of the Nottingham Trent University for the degree of Doctor of Philosophy

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Declaration

This work has not been accepted in substance for any other degree and is not concurrently being submitted in candidature for any other degree.

This is to certify that the work was carried out by the candidate himself. Due acknowledgement has been made of all assistance received.

Signed.....
(Candidate)

Signed.....
(Director of Studies)

Abstract

Collagen, type I, is a highly abundant natural protein material which has been cross-linked by a variety of methods including chemical agents, physical heating and UV irradiation with the aim of enhancing its physical characteristics such as mechanical strength, thermal stability, resistance to proteolytic breakdown, thus increasing its overall biocompatibility. However, in view of the toxicity of residual cross-linking agents, or impracticability at large scales, it would be more useful if the collagen could be cross-linked by a milder, efficient and more practical means by using enzymes as biological catalysts. We demonstrate that on treating native collagen type I with both tissue transglutaminase (TG2; tTG; guinea pig liver) and microbial transglutaminase (mTG; *Streptoverticillium mobaraense*) leads to an enhancement in cell attachment, spreading and proliferation of human osteoblasts (HOB) and human foreskin dermal fibroblasts (HFDF) when compared to culture on native collagen. The transglutaminase-treated collagen substrates also showed a greater resistance to cell-mediated endogenous protease degradation than the native collagen. Moreover, the HOB cells were shown to differentiate at a faster rate than on native collagen when assessed by measurement of alkaline phosphatase activity and osteopontin expression. It has also been demonstrated that it is possible to further enhance biocompatibility of the novel biomaterial by incorporating additional growth/attachment peptides and proteins (i.e. fibronectin) via a transglutaminase-mediated reaction. Furthermore, analyses including atomic force microscopy (AFM) confirm distinct differences; not only between the gross macro-structure of the matrix but, also, changes to the molecular formation of the fibrils of the native compared to the transglutaminase-crosslinked collagen. The results suggest that the modified collagen experiences physical conformational changes that expose new (or more) cryptic sites (consistent with a quasi-collagen-gelatin material) that leads to increased cellular characteristics brought about by enhancement of outside-in integrin mediated signaling pathways. As such, it has been demonstrated the transglutaminase enzyme can be used as a biocatalyst to modify collagen to develop novel biomaterials that are conducive to both soft and hard tissue repair applications.

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It has been an experience!

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Publications

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Patents

Chau DYS, Collighan RJ, Verderio EAM, Griffin M.
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List of Abbreviations

ADP	adenosine-5'-diphospate
AFM	atomic force microscopy
ALP	alkaline phosphatase
ATP	adenosine-5'-triphosphate
BCA	bicinchoninic acid
BSA	bovine serum albumin
Biotin-X-cadaverine	5((N-biotinoyl)amino)hexanoyl)amino)pentylamine tifluoroacetate salt
Ca^{2+}	Free calcium ion
CO_2	carbon dioxide
Coll	collagen
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme linked immunosorbant assay
ERK	extracellular signal-regulated kinases
FAK	focal adhesion kinase
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FN	fibronectin
FXIII	factor XIII
GC	gas chromatography
GDP	guanosine-5'-diphosphate
Gly	glycine
gpl	guinea pig liver
GTP	guanosine-5'-triphosphate
HFDF	human foreskin dermal fibroblast cells
HRP	horseradish peroxidase
HOB	human osteoblast cells
HYP	hydroxyproline
IgG	immunoglobulin
kDa	kilodaltons
KO	knock out
LDH	lactate dehydrogenase
M	molar
MAb	monoclonal antibody
MEF	mouse embryonic fibroblast cells
ml	milliliters
mM	millimolar
MMP	matrix metalloproteinase
µl	microliter
µM	micromolar

MOPS	3-(4-morphonyl) 1-propanesulphonic acid
mTG	microbial transglutaminase
NADH	nicotinamide adenine dinucleotide
OPN	osteopontin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PF	parafolmaldehyde
pH	negative log of hydrogen ion concentration
PLC	phospholipase C
PMSF	phenyl methyl sulfonyl fluoride
Pro	proline
R281	N-Benzyloxycarbonyl-L-phenylalanyl-6-dimethylsulfonium-5-oxo-L-norleucine)
R283	1,3-dimethyl-2[(oxopropyl)thio] imidazolium
RGD	argine-glycine-aspartic acid
RT	room temperature
SD	standard deviation
SDS	sodium dodecyl sulphate
TAE	tris-Acetate-EDTA
TCA	trichloroacetic acid
TE	tris-EDTA
TEMED	N,N,N,N'-tetramethylene diamine
TG	transglutaminase
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinase
tTG	tissue transglutaminase
TMB	3,3',5,5'- tetramethylbenzidine
Tris	tris(hydroxymethyl)-aminoethane
Triton X-100	t-ocylphenoxypolyethoxyethanol
UV	ultra violet
WT	wild type
X-link	cross-link
XTT	(2,3-bis[methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide
2-D	2-dimension
3-D	3-dimension

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INTRODUCTION

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1.1. TISSUE ENGINEERING

During the last two decades, there have been many significant and important advances within medicine and biotechnology, predominantly in the field of tissue engineering. Although the term was originally coined at a National Science Foundation (NSF) workshop in 1988, it has recently been defined as "the application of biological, chemical and engineering principles towards the repair, restoration or regeneration of living tissues using biomaterials, cells and factors alone or, in combination" [Laurencin *et al.*, 1999]. Thus, tissue engineering is an inter-disciplinary field that applies the principles of biology and engineering to a truly multi-faceted aspect - spanning from the control of cellular responses to material implants, manipulating the healing environment to control the structure of the regenerated tissue, the production of cells and tissues for transplantation for *in vivo* applications, to developing a quantitative understanding of many biological equilibrium and rate processes. Distinctively, this form of therapy differs from the standard employed therapies in that the engineered tissue, ultimately, becomes integrated within the patient, affording a potentially permanent and specific cure of the disease state [Hubbell, 1995]. Although multiple organ failure is responsible for the deaths of a large number of patients in their final days, the majority of diseases in the current population results from damage, failure, or the loss of a single organ or tissue component [Bhatia and Chen, 1999]. Similarly, mortality and morbidity due to large area, full-thickness skin burns or damage is a direct result of our inability to replace the barrier functions of skin quickly enough to prevent infection and mass complications [Bhatia and Chen, 1999]. Hence, immediate and permanent solutions need to be quickly sought in order to satisfy the rapidly increasing demand for tissue substitutes. Presently, three major types of clinical therapies are utilised in the replacement and repair of damaged tissues, namely the autograft, allograft and xenograft.

Autografting, regarded as the 'gold standard', involves harvesting tissue from a host and transplanting it to another location within the same host [Chandran, 2003]. Grafts of this type lead to accelerated healing, rapid incorporation and revascularisation. Importantly, there is no risk of immune rejection or disease

transmission from foreign tissue [Parikh, 2002; Goldberg and Stevenson, 1992 and 1994]. However, the tissue harvesting procedure involves a second surgery and introduces additional trauma including, donor-site morbidity, increased risk to the patient from additional surgery (infection and blood loss), haematoma formation, nerve injury, cosmetic defects, tumour transplantation and, frequently, resulting in chronic pain at the donor site [Ross *et al.,* 2000; Seiler and Johnson, 2000; Skaggs *et al.*, 2000; Arrlington *et al.,* 1996; Summers and Eisenstein, 1989; Cowley and Anderson, 1983]. Furthermore, it may fail in clinical practice as many of the cellular elements do not survive transplantation [Sandhu, 1999].

Allografting involves harvesting tissues/organs from a donor and transplanting it to another patient of the same species and has become popular due to the advancement in anti-rejection drugs and therapy [Carter, 1999]. However, there are also several problems and complications that may arise from their use, including: the risk of disease transmission, immunogenicity complications, potential tissue rejection, lost of biological/mechanical properties due to secondary processing, increased costs and reduced availability world-wide due to financial and religious concerns [Friedlaender *et al.,* 1999; Damien and Parsons, 1991; Bos *et al.,* 1983; Bonfiglio and Jeter, 1972]. Finally, xenografting involves harvesting the tissues from a donor of a given species and inserting it into a recipient of belonging to a different species [Aubard, 2002]. As with allografts, the potential for tissue rejection and the possible transmission of disease is magnified and remains a major concern. In addition, there are many ethical concerns regarding this use of animals as 'disposable factories'. However, the advantage of a readily available potential supply and the possibility of a standardised product make this option attractive to the medical profession. Researchers, strengthened and encouraged by the expanding strategies in DNA science, immunology and the human genome project, continue to investigate the possibility of producing transgenic animals that may be recognised as pseudo-human organs. Examples of current engineered biomaterials are shown in Figure 1.1.

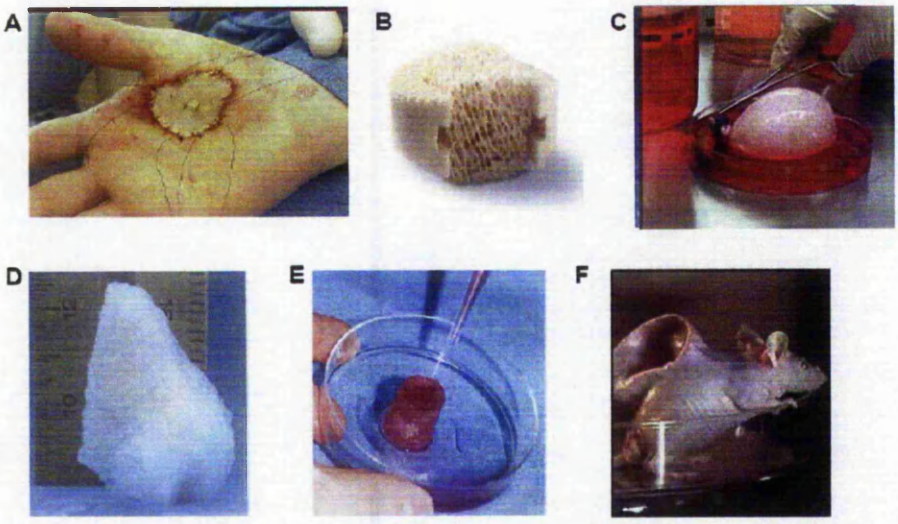

Figure 1.1. Tissue engineered substitutes and implants currently in medical use **(a)** skin grafts [www.medscape.com] **(b)** bone scaffold [www.artimplant.se] **(c)** bladder [www.dsc.discovery.com] **(d)** nose [www.turkcadcam.net] **(e)** blood vessel [www.biomed.metu.edu.tr] **(f)** ear (transgenic mouse) [www.news.bbc.co.uk]

Although it can be seen that transplant biology has advanced in the past several decades, the limited supply of donor organs and the accompanying life-long immuno-suppression or risks of cross-infection still pose major complication. Hence, one of the most innovative and promising cross-disciplinary approaches to addressing this problem involves the development of engineered tissue and organ replacement products [reviewed by Lanza *et al.*, 1997; Langer and Vacanti, 1993]. Many man-made biomimetic materials and devices have already been created by engineers and scientists to try to replicate, augment and extend functions performed by biological systems e.g. arteries [Niklason *et al.*, 1999; Black *et al.*, 1998;], bone [Isogai *et al.*, 1999; Peter *et al.*, 1998], bladder [Oberpenning *et al.*, 1999], cartilage [Binette *et al.*, 1998], heart valves [Carrier *et al.*, 1999; Mayer *et al.*, 1997], liver [Griffith *et al.*, 1997], pancreas [Colton, 1995] and nerves [Borkenhagen *et al.*, 1998, Chamberlain *et al.*, 1998].

However, the materials used for these applications are subject to general wear and tear, fracture and toxicity. Furthermore, they do not remodel with time (i.e. metal bone implants cannot grow with the patient and cannot change shape in response to the loads placed upon it and are best suited as temporary therapies

until a true donor organ becomes available [Bhatia *et al.*, 1999]. While all the aforementioned therapies have had a significant medical impact, there are newer technologies on the horizon. The next-generation of therapies include harvesting tissues and organs from genetically engineered and/or cloned animals, use of gene therapy, stem cell technology and advances in cell and tissue engineering [Bhatia *et al.*, 1999]. While cloning and gene therapies have received much publicity, there are still significant technical, economical, political and ethical issues that must be overcome before they can be used. Contrastingly, many of the tissue-engineering approaches hold the promise of providing more immediate solutions: by assembling cells and scaffolds into engineered tissue, it is hoped that these implants can virtually replace the functions of the damaged tissue and be available on demand. Eventually, tissue engineered medical products may be created that even outperform our natural tissues.

Tissue engineering has all the advantages of graft therapies and is not limited with regards to supply- the required cells or tissues may be expanded prior to re-implantation. In addition, this technique avoids morbidity at any donor sites because only a small biopsy is required to obtain the cells for amplification *in vitro*. A key component of 'modern' tissue engineering is the control of the growth, differentiation and behaviour of selective cells on biological substrates, facilitating their organisation into functional tissue or organs [Hubbell, 1995]. As yet, only three general approaches, as summarised by the Pittsburgh Tissue Engineering Initiative (PTEI), have been adopted for the creation of new tissue, namely:

1. **Design and growth of tissue externally for later implantation to repair or replace diseased and/or damaged tissues.** By growing tissues outside the body, the cost of tissue harvest, surgical costs and post-operative patient costs would be significantly reduced. The most common example is the skin graft that is used for treatments of burns

2. **Implantation of cell-containing or cell-free devices that induce the regeneration of functional human tissues.** This approach relies on the purification and large-scale production of appropriate 'signal' molecules, such as growth factors, or biologically active peptides and motifs which assist the

assembly of 3-D configurations, to which the cells attach and grow to reconstitute tissues.

3. **The development of external or internal devices containing human tissues designed to replace the function of diseased internal tissues.** This approach involves isolating cells from the body, using such techniques as stem cell therapy, placing them on or within structural matrices, and implanting the new system inside the body or using it outside the body. Examples, of this approach include the repair of bone, muscle, tendon and cartilage, as well as the cell-lined vascular grafts and development of an artificial liver.

1.1.1. Biomaterials

A biomaterial is used to make devices to replace a part or a function of the body in a safe, reliable, economic and physiologically acceptable manner [Hench and Erthridge, 1982]. A variety of devices and materials presently used in the treatment of disease or injury include such commonplace items such as sutures, needles, catheters, plates, tooth fillings etc. Over the years, various definitions of the term biomaterials have been proposed. For example, Black defined biomaterials as a 'nonviable material used in a medical device, intended to interact with biological systems [Black, 1992]. Other definitions have included 'materials of synthetic as well as of natural origin in contact with tissue, blood and biological fluids, and intended for use for prosthetic, diagnostic, therapeutic and storage applications without adversely affecting the living organism and its components' [Bruck, 1980]. By contrast, a biological material is a material such as skin or artery produced by a biological system. Artificial materials that are simply in contact with the skin, such as hearing aids and wearable artificial limbs are not included in our definition of biomaterials since the skin acts as a barrier with the external world [Park and Bronzino, 2003]. The performance of materials in the body can be classed in many ways. Firstly, biomaterials may be considered from the point of view of the problem area that is to be solved or, be considered the body on a tissue or organ level Table 1.1, or as a systems level, Table 1.2, respectively. It should be evident from any of these perspectives that most current

applications of biomaterials involves structural functions even in those organs and systems which are not primarily structural in their nature, or very simple chemical or electrical functions. Complex chemical functions such as those of the liver and complex electrical or electrochemical functions such as those of the brain and sense organs cannot be carried out by biomaterials at this time.

Table 1.1. Classification of biomaterials according to their target area or intended organ [adapted from Park and Bronzino, 2003]

Application	Example	Organs	Example
Replacement of diseased or damaged part	Artificial hip joint, kidney dialysis machine	Heart	Cardiac pacemaker, artificial heart valve, total artificial heart
Assist in healing	Sutures, bone plates and screws	Lung	Oxygenator machine
Improve function	Cardiac pacemaker, intraocular lens	Eye	Contact lens, intraocular lens
Correct functional abnormality	Cardiac pacemaker	Ear	Artificial stapes, cochlea implant
Correct cosmetic problem	Augmentation mammoplasty, chin augmentation	Bone	Bone plant, intramedullary rod
Aid to diagnosis	Probes and catheters	Kidney	Kidney dialysis machine
Aid to treatment	Catheters, drains	Bladder	Catheter and stent

Table 1.2. Biomaterials classified according to body system [Park and Bronzino, 2003]

System	Examples
Skeletal	Bone plate, total joint replacement
Muscular	Sutures, muscle stimulator
Circulatory	Artificial heart valves, blood vessels
Respiratory	Oxygenator machine
Integumentary	Sutures, burn dressings, artificial skin
Urinary	Catheters, stent, kidney dialysis machine
Nervous	Hydrocephalus drain, cardiac pacemaker, nerve stimulator
Endocrine	Microencapsulated pancreatic islet cells
Reproductive	Augmentation mammoplasty, other cosmetic replacements

1.1.2. Scaffolds

The fundamental principle for tissue engineering involves growing the appropriate cells *in vitro* for the desired tissue/organ before inserting into the body of the host. These implantable parts/devices exploit biological components, referred to as biological constructs, or scaffolds, and provide the initial ECM required to support the cells as well as potentially defining the micro- and macro-structure of the final engineered structure [Goessler *et al.*, 2003; Sachlos et al., 2003]. So far, several parameters have been identified as crucial for the production of suitable tissue engineering scaffolds: (i) appropriate surface chemistry to favour cellular attachment, differentiation and proliferation; (ii) be developed from biodegradable, bioresorbable or biocompatible material; (iii) possess the required physical and mechanical characteristics to suit the intended application; (iv) easily manipulated and manufactured and (5) possess high interconnecting porosity to favour tissue integration, cellular migration and vascularisation [Goessler *et al.*, 2003; Hutmacher, 2001 and 2000; Bhatia and Chen, 1999]. It is apparent that many materials are utilised in tissue engineering as scaffolds for the cellular components of tissue-engineered constructs and are summarised in Table 1.3.

Table 1.3. Materials for use in the body [adapted from Park and Bronzino, 2003]

Materials	Advantages	Disadvantages	Examples
Polymers (nylon, silicone rubber, polyester, polytetrafuoroethylene, etc.)	+ resilient + easy to fabricate	- weak - deforms with time - may degrade	Sutures, blood vessels, hip socket, ear, nose, other soft tissues
Metals (Ti and its alloys, Co-Cr alloys, stainless steels, Au, Ag, Pt, etc.)	+ strong and tough + ductile	- may corrode - dense - difficult to make	Joint replacements, bone plates and screws, dental root implants, pacer and suture wires
Ceramics (aluminium oxide, calcium phosphates including hydroxyapatite, carbon)	+ very biocompatible + inert + strong in compression	- brittle - not resilient - difficult to make	Coating of dental and orthopaedic implants, femoral head of hip replacement
Composites (carbon-carbon, wire or fibre-reinforced bone cement)	+ strong + tailor-made	- difficult to make	Joint implants, heart valves
Biologically-derived materials, proteins and polysaccharides (fibrin, chitosan, hyaluronic acid, collagen, etc.)	+ biological active and compatible	- weak - limited source - immuno-response	Soft and hard tissue repair and replacements, ophthalmology, grafts

An ideal scaffold must provide the mechanical framework required to form a complex structure, as well as allowing the appropriate cell behaviour and signalling. Furthermore, it must also provide an environment whereby the cells are able to maintain their phenotype and synthesise or express the required proteins and molecules for that specific tissue function [Goessler *et al.*, 2003; Bhatia and Chen, 1999]. Currently, research is rapidly evolving towards devloping specialised biomaterials that can: erode into naturally-occurring by-products (i.e. temporary scaffold), be incorporated/impregnated with specific biological factors to direct tissue growth, allow cellular colonisation or trigger a host reaction (drug delivery device), block undesirable biological responses (barrier), and alter their material mechanical properties in response to an external environmental stimuli (smart materials) [Pachence and Khon, 1997; Hubbell, 1995].

1.1.3. Biocompatibility

The complications frequently seen during the use of incompatible implant materials usually involve diverse host responses which lead to the rejection of the implant or the formation of fibrous protein capsules that enclose the implants [Kantlehner *et al.*, 2000]. Although the development of biomaterials has focused on their mechanical characteristics, it has become clear that it is simply not enough to minimise undesirable tissue-biomaterial interactions but, also necessary to enhance and promote beneficial functions in the surrounding cells and tissues of the implant. Advances in this field have already been made with the development of biocompatible, biodegradable synthetics polymers, which allow cells to colonise and deposit their own extra-cellular matrix (ECM), before eventually being degraded and leaving newly formed tissue behind [Verderio *et al.*, 2001]. Most currently employed materials used as tissue engineering implants are generally regarded as biocompatible; that is, they are non-toxic and stable against degradation *in vivo*. Unfortunately, these materials only allow a limited degree of integration with the surrounding tissue to occur and, as such, it has been identified that the limited acceptance of materials is due to improper (biological and physical) contact between the implant surface and the cells of the regenerating tissue [Kantlehner *et al.*, 2000]. However, biocompatibility, when strictly defined is the ability of a material to elicit an appropriate biological

response in a given biological application [Wataha, 1999]. This definition implies that any material placed into the body will not be inert and will interact with tissues in a dynamic way, altering both the material and the tissues around it. Unfortunately, the interfacial reactions that exist between the biomaterials and living tissues are poorly understood but deemed critical to identify the key factors that enhance the recruitment and colonisation of host cells that encourage tissue repair [Stephansson *et al.*, 2002].

The current literature specifies that the initial attachment of a cell to a substratum is mediated by cell-surface adhesion factors, among which the cytoskeleton-linked integrin receptors are the most documented. The re-organisation of the cytoskeleton and the ECM, following attachment of cells, results in their flattening, spreading and stimulation of a number of intracellular signalling pathways. Ultimately, a wide range of biological events including cell proliferation, migration, and differentiation can be forthcoming. [Zhu *et al.*, 1996; Streuli *et al.*, 1991; Adams and Watt, 1990; Werb *et al.*, 1989; Menko and Boettiger, 1987]. Hence, a current approach is to render the surface of the implant bioactive based on the incorporation or, even enrichment, with cell adhesion factors, which may promote and enhance cell attachment, spreading, increase the formation of focal contacts and cytoskeletal organisation, thus influence cell survival and differentiation on the matrices [Verderio *et al.*, 2001]. In many of these biomaterial applications, the cells interact with proteins either adsorbed from physiological fluids, synthesised by the cells themselves, or, by means of peptides that have been artificially introduced or impregnated on the surface [Hynes, 1992].

Synthetic and biologically derived polymers play a crucial role in the design and engineering of new therapeutic and diagnostic devices for medical applications such as tissue regeneration and replacement (i.e. tissue engineering). Synthetic polymers have been obvious candidates for such applications because their chemical and physical properties can be controlled. However, these materials often lack the ability to signal (orchestrate) fundamental cellular processes including adhesion, migration, differentiation and proliferation. With hopes of increased biocompatibility and elicitation of a desirable cellular response, biomaterials have been also fashioned from tissues and biologically derived

molecules (e.g. collagen and glycosaminoglycans). In fact, many of the molecules that compose the extracellular matrix (ECM) of tissues exhibit the ability to polymerise and form complex, three-dimensional (3D) supramolecular assemblies in vitro, a process known as 'self-assembly' [Brightman *et al.*, 2000].

1.2. COLLAGEN

Collagen is the most ubiquitous and abundant structural protein in vertebrates, accounting for about 20-30% of the total protein [Harkness, 1961]. It is present in all tissues and organs of the body with its function ranging from serving crucial biomechanical functions in skin, bone, tendon and ligament to controlling cellular gene expressions in development [Nimni and Harkness, 1988]. Collagen emerged at the early stages of evolution in primitive animals such as jellyfish, coral and sea anemones but, with higher organisms, synthesis is usually associated predominantly with fibroblasts, which originate from pluripotential adventitial or reticulum cells [Bergeon, 1967]. Earlier investigations such as amino acid content determination [Eastoe, 1967], x-ray diffraction analyses [Rich and Crick, 1961], electron microscopy and the general physicochemical composition have conclusively established the structure of collagen molecules [Ramachandran and Sasisekharan, 1965; Burge, 1964; Gross, 1963]. The "collagens" are a structurally related multifunctional family of proteins that form supramolecular assemblies within the ECM. They are unique in structure, size, their amino acid sequence and composition and constitutively express one or more domains of the exclusive triple helical motif, known as the "collagenous domain" [Rao, 1995; Miyata *et al.*, 1992]. This conserved region consists of a right-handed triple helical coil comprising three polypeptide chains known as α-chains. Collagen can be identified as a homotrimer, whereby all three chains are identical, or as a heterotrimer where the chains are considered different- consisting of either 2 or 3 dissimilar α-chains. Each α-chain in the molecule is coiled to an extended left-handed polyproline II-type helix, then into the three left-handed helical α-chains before being intertwined to one another and folded into a right-handed triple-helical structure [Bateman *et al.*, 1996] as shown in Figure 1.2.

The triple helical structure is stabilised by the high content of proline (Pro) and hydroxyproline (Hyp) via covalent bonding. Moreover, it is the presence of Hyp that is regarded as vital to the gross structure as it provides the required hydrogen bonding between the chains, between adjacent carboxyl and amino groups, to further stabilise the configuration [Harkness, 1966]. In order for the α-chains to form the distinct collagen triple-helix, a repeating sequence of amino acids of $[Gly\text{-}X\text{-}Y]_n$ is required, where X and Y can be any amino acid but often X is Pro and Y is Hyp. Every third amino acid is located in the centre of this triple helix structure, a very small and restricted space, of which only the smallest amino acid, glycine (Gly), can fit.

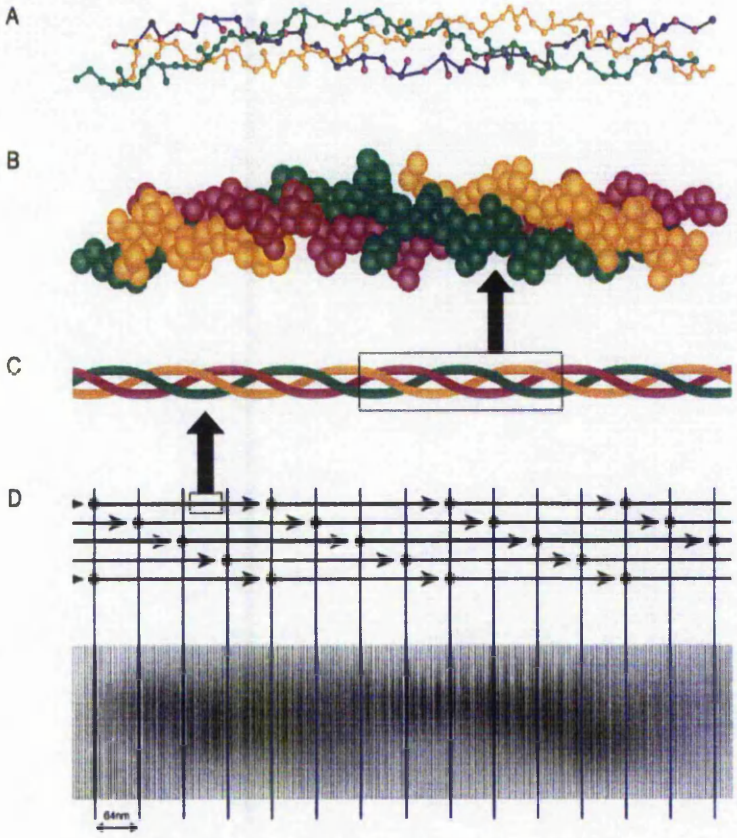

Figure 1.2. Overall structure of collagen. (A) schematic residue drawing (B) van der Waals model of the helical structure of collagen (C) three single chains intertwined into a triple-stranded helix (D)collagen molecules aligned in D-period staggered formation in a fibril producing overlap and hole regions based on typical X-ray diffraction pattern [adapted from http://wwwfac.mcdaniel.edu using Adobe Photoshop®]

Hence, the principle feature that affects the helix formation is the high content of glycine and prolyl amino acid residues [Piez, 1984]. The typical amino acid content of collagen can be seen in Table 1.4.

Table 1.4. Typical amino acid content of collagen

Amino acid	Content (residues/1000 residues)*
Gly	334
Pro	122
Hyp	96
Acid polar (Asp, Glu, Asn)	124
Basic polar (Lys, Arg, His)	91
Other	233

* Reported values are average values of 10 different determinations for tendon tissue [Eastoe, 1967]

Thus, if mutations of the residues in the triple helical domain occur, severe disruptions of the collagen molecules can be seen, usually resulting in connective tissue abnormalities such as osteogenesis imperfecta [Byers, 2000; Forlino and Marini, 2000; Prockop and Kivirikko, 1995]. As such, this triple-helical sequence and structure has been highly conserved during evolution, resulting in the similarity of the different collagens expressed by different species. However, it is the difference in the telopeptide ends, derived from a variation in the amino acid sequences, between human and mammalian collagens that are responsible for most of the immunological responses experienced [Geiger and Friess, 2002].

Under physiological conditions, the individual collagen molecule is rod-shaped with a length and diameter of approximately 300nm and 1.5nm, respectively, and has an approximate weight of 300kDa [Nimni and Harkness, 1988; Traub and Piez, 1971]. These basic collagen molecules are, themselves, capable of aggregating (longitudinally and bilaterally) to form highly ordered and staggered microfibrils which then form into fibrils. The two-dimensional (2-D) structure of a collagen fibril has been previously clearly identified [Hodge and Petruska, 1963; Bear, 1952]. In this structure, the collagen molecules are staggered with respect to one another by a distance of D (64-67nm) or multiple of D, where D is the fundamental repeat distance and seen as a dark and light banded pattern [Li,

2003]. It is also commonly known as the D-period and, since a collagen molecule has a length of about 4.4D, this staggering of collagen molecules creates overlap regions of about 0.4D and holes or defect regions of about 0.6D of which the collagen fibrils then organise in to fibres which can then form even larger fibre bundles [Geiger and Friess, 2002].

1.2.1. Collagen superfamily

It has now been ascertained that collagen is encoded by a family of at least 38 genes and that over 19 different types of collagen exist- of which distinct variations and structural complexity exists e.g. physical characteristics, number of collagenous domains, fibril assembly and organisation, splice variants, post-translational modifications, biological functions, tissue distribution and localisation [Lee *et al.*, 2001; Myllyharju and Kivirikko, 2001; Byers, 2000; Prockop and Kirivikko 1995; Fukai *et al.*, 1994]. This collagen superfamily can be classified into three major groups and several different subgroups, based on their assembly and physical characteristics. The collagen types I, II, III, V and XI are known as the "fibril forming collagens" and are regarded as the major members of the collagen family with regards to their abundance and ability to form fibres in the ECM [Li, 2003; Yamauchi, 2003; Lee *et al.*, 2001]. These molecules share large regions of homologous sequences (independent of species) and are composed of three domains: a short amino-terminal non-triple helical (N-telopeptide) domain, a long central uninterrupted triple helical domain and a short carboxyl-terminal non-triple helical domain (C-telopeptide) [Timpl, 1984]. It has thus been deduced, due to the similarities of the nine genes that encode the α-chains of these collagens, that they originally arose from multiple duplications from a single ancestral gene [Vuorio and de Crombrugghe, 1990]. Typical chain compositions are given in Table 1.5. It should also be noted that only minor differences exist between collagens from different vertebrate species [Timpl, 1984].

The second group is known as the "Fibril-Associated Collagen with Interrupted Triple helices" (FACIT) or, sometimes, just "fibril-associated collagens" which include the collagen types IX, XII, XIV, XVI, XIX, XX and XXI [Yamauchi, 2003; Lee *et al.*, 2001]. These contain at least two short triple helical domains that are

interrupted by short specific non-collagenous regions [Olsen *et al.*, 1995]. Members of this group do not form fibrils themselves but attach to the surface of pre-existing collagen fibrils by exposing their terminal amino domains out of the fibrils and into the proposed matrix, and are considered as 'connectors' between fibrils or between fibrils and other substrates or cells [Yamauchi, 2003].

Table 1.5. Collagen chain composition and body distribution [adapted from Friess, 1998]

Collagen type	Chain composition	Tissue distribution
I	$(\alpha1(I))_2\alpha2(I)$, trimer $(\alpha1(I))_3$	Bone, cornea, dentin, dermis, fibrocartilage, intestine, large vessels, skin, tendon
II	$(\alpha1(II))_3$	Hyaline cartilage, notochord, nucleus pulposis
III	$(\alpha1(III))_3$	Dermis, heart valve, gingival, intestine, large vessels, uterine wall (usually coexists with type I in bone, tendon, cornea)
IV	$(\alpha1(IV))_2\alpha2(IV)$	Basement membranes
V	α1(V)α2(V)(3(V) or $(\alpha1(V))_2\alpha2(V)$ or $(\alpha1(V))_3$	Bone, cornea, gingival, hyaline cartilage, large vessels, placental membranes
VI	α1(VI) α2(VI) α3(VI)	Descemet's membrane, heart muscle, nucleus pulposus, skin
VII	$(\alpha1(VII))_3$	Cartilage, cornea, lung, placenta, skin
VIII	α1(VIII)α2(VIII) chain organisation of helix unknown	Produced by endothelial cells. Descemet's membrane
IX	α1(IX)α2(IX)α2(IX)α3(IX)	Cartilage
X	$(\alpha1(X))_3$	Hypertrophic and mineralising cartilage
XI	$1\alpha2\alpha3\alpha_1$ or α1(XI)α2(XI)α3(XI)	Cartilage, intervertebral disc, vitreous humour
XII	$(\alpha1(XII))_3$	Bovine periodontal ligament, chicken embryo tendon
XIII	unknown	Bone intestinal mucosa, fetal skin

The final group are deemed "non-fibrillar" collagens as they are unable to form fibrils or associate closely with members of the collagen superfamily [Yamauchi, 2003]. The members of this group can be subdivided further into: (1) network forming collagens that include the basement membrane collagen (type IV) and hexagonal network-forming collagens (types VIII and X), (2) the anchoring fibril collagen (type VII), (3) microfibrillar collagen (type VI), (4) the "multiplexins" (Multiple triple helix domains with interruptions) which include collagens type XV, XVIII, (5) the transmembrane collagens (types XIII, XVII) [Samuel *et al.*, 1998].

1.2.2. Collagen for medical applications

The major characteristics that are exploited when collagen is considered for medical applications is summarised in Table 1.6. Collagen is a good surface-active agent, capable of penetrating lipid-free interfaces, resulting in its ease of adsorption by the body [Fonseca *et al.*, 1996], as well as expressing weak antigenicity [Maeda *et al.*, 1999]. Not only is collagen biodegradable and biocompatible, it possesses high tensile strength, stability and has a great affinity for water, identified by its haemostatic ability [Maeda *et al.*, 1999; Friess, 1998]. Above all, the key aspect for the usefulness of collagen in tissue engineering is that collagen is capable of forming fibrils and/or fibre bundles with increased strength and stability through self-aggregation and/or, additionally, *in vitro* as well as *in vivo* cross-linkages. As a consequence, a large variety of different forms of collagen preparations exists, summarised in Table 1.7 with applications ranging from dermatology, neurosurgery, ophthalmology, gynaecology to orthopaedic surgery. However, even though collagen is regarded as stable due to its native function as a structural protein or, even following enhanced cross-linking, it is still liable to enzymatic degradation by collagenases, other telopeptide-cleaving and cross-linkage/site-specific proteolytic enzymes [Woolley, 1984].

Table 1.6. Advantages and disadvantages of collagen as a medical biomaterial

Advantages	Disadvantages
+ abundant and easily isolated and purified from source	- High cost of pure collagen type
+ ideal physiochemical properties (tensile strength, stress, strain etc.)	- batch-to-batch variation (e.g. cross-linkage, density, fibre size, trace impurities etc)
+ non-antigenic and non-toxic (minimal immunological response to leaching)	- Time-consuming isolation procedure with complex handling properties
+ biodegradable, bioreabsorbable and biocompatible	- Variability in enzymatic degradation rate as compared with hydrolytic degradation
+ synergic and compatible with other bioactive components and/or synthetic polymers	- Hydrophilicity which leads to swelling and more rapid release
+ easily modifiable: by addition of cross-linkages or by combining with other biological molecules to improve strength and biocompatibility	- Risk of BSE and complications due to mineralisation
+ haemostatic: promotes blood coagulation	
+ capable of being formulated in variety of physical forms and or concentrations (solutions)	

Sources: Maeda *et al.*, 1999; Freiss, 1998; Fujioka *et al.*, 1998; Rao, 1995; Jerome and Ranshaw, 1992

Collagen can be processed into a wide range of different physical forms, such as sheets, sponges, tubes, powders, gels and injectable solutions, all of which have found use in medical applications ranging from ophthalmological application [DeVore, 1995; Wedge and Rootman, 1992; Rubin *et al.,* 1973], wound and burn dressing [Lee *et al.*, 2001], tumour treatment and scaffolds for tissue engineering [Khor, 1997; Hubbell, 1995; Fu Lu *et al.,* 1991; Gorham, 1991; Chvapil *et al.,* 1973]; drug delivery and release mechanisms [Ning *et al.,* 1996; Davidson *et al.,* 1995; Kanekal *et al.*, 1995; Sahai *et al.,* 1995; Yu *et al.,* 1995; Sutton *et al.*, 1990; Marty *et al.,* 1978]. In short, the number of applications and successful commercial products available for the general consumer is growing year by year and are summarised in the Tables 1.7 and 1.8.

Although the use of collagen derived from animal sources raises the possibility of triggering an immunological response or even stimulating an auto-immune response if a cross-reaction with human collagen occurs, adverse reactions have only been restricted to localised redness and swelling [Webster *et al.*, 1984]. Only a few cases of clinical reactions to collagen have ever been reported and involve IgE-mediated-allergic reactions to bovine collagen [Mullins *et al.,* 1996]. It has been demonstrated that simple processing steps can also be used to reduce the immunological response of collagen: enzymatic or alkaline purification methods are used to cleave the species-specific ends of collagen, additional exogenous cross-linking has also been seen to reduce immunological potential- with the additional bonus of strengthening the collagen [Geiger and Friess, 2002]. Even though yet unknown and adverse reactions to collagen may exist, the overwhelming positive use of collagen in tissue engineering and many other therapeutic applications drives a continuous effort in the research and development of collagen-based biomaterials. Although the immunological data regarding the use of these collagen-based biomaterials is not as comprehensive as other implant materials, no collagen-induced adverse immunological response to non-allograft dermal substitutes have been documented, despite the numerous collagen sources and extraction methods used [Lynn *et al.,* 2004]. The immunological observation of several commercial collagen-based products and their clinical trials are summarised in Table 1.9.

Table 1.7. Physical forms of collagen and their application in medicine [adapted from Chvapil, 1979]

Form of collagen	Application	References*
Solution or gel	Plasma volumiser and expander, delivery vehicle for controlled-release drug systems, cosmetic surgery (lips, skin), vitreous body, tear insert replacement	Walsh *et al.*, 1999; Bloomfield *et al.*, 1977; Pruett *et al.*, 1974; Dunn *et al.*, 1969
Flour or powder	Haemostatic agent, bone-repair and fracture substitute	Mergenhagen *et al.*, 1973
Fibres	Surgical wound dressing: sutures, weaving blood vessels, valve prostheses, haemostatic fleece	Okada *et al.*, 1992; Dimitrova, 1977
Film, membrane or tape	Corneal replacement, contact lens, haemodialysis, artificial kidney, membrane oxygenators, surgical wound dressing: pads, patches (aneurism, bladder, hernia), cosmetic surgery (skin), urothelial grafts, collagen membrane for *Dura Mater* substitute, replacement of tympanic membrane	Orwin and Hubel, 2000; Damour *et al.*, 1998; Sabbagh *et al.*, 1998; Trent and Kirsner, 1998; Frenkel *et al.*, 1997; Toolan *et al.*, 1996; Hansen *et al.*, 1974; Stenzel *et al.*, 1969; Rubin *et al.*, 1968; Kucera *et al.*, 1966; Jannetta and Whayne, 1965; Kline, 1965; Salen and Simbach, 1965; Bellucci and Wolff, 1964
Sponge or felt	Surgical wound dressing: pads, weaves, tissue replacement, bone-cartilage substitute, contraceptives (barrier method), reservoir for controlled-release drug, artificial liver and soft tissue repair/replacement	Ranucci *et al.*, 2000; Walsh *et al.*, 1999; Girdler, 1998; Glowacki *et al.*, 1998; Frenkel *et al.*, 1997; Toolan *et al.*, 1996; van Wachem *et al.*, 1996; Heermeier *et al.*, 1995; Schoeters et al., 1992
Tubing	Vessel prostheses, reconstructive repair and/or replacement of hollow organs (oesophagus, trachea), protective wrapping of nerves	Dardik *et al.*, 1974; Metz and Seeger, 1969; Braun, 1966; Krajicek *et al.*, 1964
Composite (collagen/synthetic polymer; collagen/biological polymer; collagen/ceramic)	Vascular repair, skin repair, wound dressing, soft-tissue augmentation, hard-tissue repair	Li, 2003

* only a small representative of references are given

Table 1.8. Commercialised products and their applications in biomedical surgery [adapted from Li, 2003]

Applications	Commercial applications
Cardiovascular surgery and cardiology	Vascular grafts [Jonas *et al.*, 1988; Li, 1988; Dardik *et al.*, 1974]; Sawyer *et al.*, 1977]; heart valves [Angell *et al.*, 1982; Walker *et al.*, 1983]; vascular repair [Merino *et al.*, 1992]
Dermatology	Injectable collagen for soft tissue augmentation [Webster *et al.*, 1984]; skin substitutes [Bell *et al.*, 1981; Yannas and Burke, 1981] Wound dressings [Armstrong *et al,* 1986]
Haemostasis	Sponge and fibre structures used in cardiovascular [Abbott and Austin, 1975]; neurosurgical [Rybock and Long, 1977]; dermatological [Larson, 1988]; orthopaedic [Blanche and Chaux, 1988]; and oral surgical applications [Stein *et al.,* 1985]
Neurosurgery	Nerve enclosure [Archibald *et al.,* 1991; Yannas *et al.*, 1985]
Ophthalmology	Corneal shield or graft [Ruffini *et al.*, 1989; Dunn *et al.*, 1967; Reidy *et al.*, 1990]; vitreous substitute [Dunn *et al.,* 1969]. Tear duct replacement [Bloomfield *et al.*, 1977]
Orthopaedic surgery	Bone marrow replacement [Hollinger *et al.*, 1989]; meniscus regeneration [Li *et al.*, 1994]; regeneration of Achilles tendon [Kato *et al.*, 1991]
Periodontal and oral surgery	Periodontal ligament regeneration [Blumenthal, 1988]; oral wound dressings [Ceravalo and Li, 1988]; augmentation of alveolar ridge [Gongloff and Montgomery, 1985]
Other applications	Drug delivery support [Sorensen *et al.*, 1990]; delivery vehicles for growth factors and bioactive macromolecules [Deatherage and Miller, 1987; Li *et al.*, 1996]; tissue engineering scaffold [Bell *et al.*, 1981]

Table 1.9. Immunological observations from selected clinical trials [adapted from Lynn *et al.*, 2004]

Composition	Type	Extraction	X-linking	Product (trials)	Application	Immunological response
Collagen	Bovine type I (95%), type III (5%)	Pepsin	-	Zyderm (>1000000)	Injectable soft tissue augmentation	Pre-existing bovine collagen allergy in 2% of patients: 1% developed allergy in response to implant; adverse reaction to implant (localised inflammation, granuloma formation) in 1% of patients
Collagen	Bovine type I	Pepsin	-	Atelocollagen (705)	Injectable soft tissue augmentation	Pre-existing bovine collagen allergy in 3.8% of patients; adverse reaction to implant (localised inflammation) in 2.3% of patients
Collagen-GAG/silicone	Bovine type I	Acid	Glut, DHT	Integra (159)	Skin substitute for wound closure	No adverse reactions; specific immunological data not present
Keratinocytes/ collagen and fibroblasts	Bovine type I	Acid	-	Apligraf (107)	Skin substitute for wound closure	Pre-existing bovine collagen allergy in 3% of patients: no patients developed allergy in response to implant; no adverse reactions of any kind observed in response to grafts
Collagen/Hap/TCP	Bovine type I	Pepsin	-	Collagraft Zimmer (303)	Bone filler for spinal fusion, fracture fixation	Postoperative development of bovine collagen allergy observed in 0.33% of patients; no associated complications
Collagen/Hap	Bovine type I	n/a	n/a	Alveoform Collagen (77)	Bone filler for maxillae and mandibular augmentation	Pre-existing bovine collagen allergy in 6.5% of patients: additional 6.5% developed allergy postoperatively; no adverse affect on surgical outcome
Collagen/thrombin	Bovine type I	Pepsin	-	CoStasis Cohesion (92)	Sprayable surgical haemostat	Pre-existing bovine collagen allergy in 1% of patients: additional 8% developed allergy in response to implant; no adverse reaction on operative outcome
PGA tube/collagen filler	Porcine type I (85%), type III (15%)	Pepsin	DHT	Nerve-Reg Conduit (non-commercial) (65)	Digital peroneal nerve grafting	No adverse reactions; specific immunological data not presented

GAG = glycosaminoglycan; Hap – hydroxyapatite; TCP = tricalcium phosphate; Glut = glutaraldehyde; DHT = dehydrothermal treatment

1.2.3. Collagen type I

As described earlier, the collagens express enormous diversity in their structure, function and location. Amongst the broad range of the collagens, the most widely used form of collagen for medical purposes is collagen type I, which accounts for approximately 30% of total body protein in mammals and provides the tensile strength, form and cohesiveness required in tissues and organs such as bone, skin, dentine, tendon and blood vessels [Geiger and Friess, 2002]. Type I collagen exists predominantly as a heterotrimeric molecule composed of two α1 chains and one α2 chain consisting of three identifiable parts: the opposing NH_2-terminal and COOH-terminal non-triple helical telopeptides (also known as the N- and C-telopeptides, respectively), and the central triple helical domain which, in fact, represents more than 95% of the total molecule and maintains the amino acid sequence of [Gly-X-Y] [Li, 2003]. The telopeptide ends, approximately 9-26 residues, make up the remaining 5% of the molecule and do not posses the [Gly-X-Y] sequence and, therefore, not triple-helical [Miller, 1984]. These three left-handed helical polypeptide chains are co-intertwined to form a right-handed helix around a central molecular axis. The two, identical, α1-chains consist of 1056 amino acid residues, with the α2-chain containing only 1029 amino acid residues [Miller, 1984]. Overall, the triple-helical structure has a rise of 0.286nm per residue and a unit twist of 108°, with 10 residues in three turns and a helical pitch (repeating distance within a single chain) of 30 residues or 8.68nm [Fraser *et al.*, 1983]. The whole molecule, also known as tropocollagen, has a length of approximately 280nm, a diameter of about 1.5nm, a molecular weight of 283kDa and has a confirmation similar to a rigid rod [Li, 2003].

The precursor molecule, known as procollagen, is secreted outside the cell before being enzymatically cleaved at both the C- and N- terminal ends to yield the final collagen molecule [Yamauchi, 2003]. The exact mechanism for the biosynthesis of type I collagen from gene transcription, self-assembly, formation of macro-structures and onto the secretion in the ECM is a very complicated, multi-step process (involving more than 15 enzymes and associated chaperones) and also requires organised coordination of several inter-related biochemical events in and outside the cell [Bateman *et al.*, 1996]. In short, the process involves four major steps: (1) gene transcription and mRNA processing, (2) transportation of the

mRNA to the endoplasmic reticulum, where several processes occur, including the translation to pre-procollagen α-chains, cleavage of the signal peptides, numerous post-translational modifications, the three chain coupling followed by the triple-helical procollagen folding, (3) transportation to the golgi apparatus, secretion in to the ECM and post-extracellular processing, (4) fibril self-assembly and further enzymatic modifications including formation of intra- and inter-molecular cross-linkages [Yamauchi, 2003].

Collagen does not exist as isolated monomers in the extracellular space within the body, but aggregates as outlined earlier into larger structures known as fibrils which are important as structural building blocks *in vivo*. Not only does the diameter of these fibrils vary in size from 50-300nm but they are also found to be arranged in specific orders both longitudinally and in cross-section depending on the tissue location, localisation and age of the host [Katz and Li, 1972; 1973]. This variation in the packing of the collagen molecules in the differing tissues is believed to be as a consequence of the intermolecular interactions involving the electrostatic and hydrophobic interactions [Hofmann and Kuhn, 1981; Katz and Li, 1981; Li *et al.*, 1975], with the terminal three-dimensional structure arranged in a hexagonal arrays. [Fraser *et al.*, 1983; Katz and Li, 1972, 1981; Miller, 1976; Yamuchi *et al.*, 1986]. As discussed earlier, there are very few interspecies differences of the collagen molecule and this consistent homology may explain why this collagen obtained from different animal species is acceptable as a material for human implantation [Li, 2003].

Major emphasis is laid on the well-known physical and mechanical properties of collagen for the formulation of biomaterials in medical surgery. Collagen has to be initially sourced and isolated from collagen-rich tissues such as skin or tendon, following which an extensive procedure is required to remove all non-collagenous materials and other contaminating structures which are the major sources of an immunological response. Thus, the final product of many standardised collagen isolation and purification products, which are commonly based on enzymatic degradation of the non-collagenous components, are mostly monomeric collagen molecules [Chvapil, 1979]. These, when reconstituted in to the desired biomaterial state, would clearly posses a much lesser degree of mechanical strength of the

final structure as compared to the native formation. Only a few purification procedures preserve the native formation of the collagen fibres and fibrils and it has been ascertained that the presence of pure native collagen fibres is a critical contribution to the final mechanical strength of the bio-implant [Yamuchi, 2003; Geiger and Friess, 2002]. Additionally, the presence of the fibrillar form of the collagen allows further orientation of these fibres into a perpendicular direction during the extrusion of the mass and, thus, further enhances the mechanical stability of the overall structure [Chvapil and Krajicek, 1970].

Moreover, for certain functions such as tissue regeneration and matrix/scaffold applications, the use of native collagen may be inadequate: that is the material may have to be customised to meet the specific application e.g. a collagen-based haemostat is only required until blood-clot formation has occurred, whereas for tissue engineering applications, the implant not only has to maintain its physical and mechanical properties, but also allow the gradual replacement by host collagen [Yamauchi, 2003; Li, 2003; Gao and Lindholm, 1996; van Wachem *et al.*, 1994]. Thus, to overcome these problems, additional cross-linking by the introduction of intra- and inter-molecular covalent bonds are introduced to the collagen preparations for further (and/or enhance) mechanical and chemical stabilisation. The exogenous cross-linking of collagen material is usually performed via a large variety of chemical agents or certain physical treatments, as summarised in Table 1.10.

Table 1.10. Treatments for the introduction of additional cross-links to native collagen [adapted from Geiger and Friess, 2002]

Chemical agents	**Physical treatment**
Aldehydes (glutaraldehyde, formaldehyde)	Dehydrothermal treatment (DHT)
Acyl azide method	Ultraviolet irradiation
Carbodiimides	Gamma irradiation
Hexamethylene diisocyanate	Microwave irradiation
Polyepoxy compounds	
Polyether oxide	
Adipyl chloride	
1,4-butanediodiglycidyl ether	
Dye-mediated photo-oxidation	
Tannic acid (chromium tanning)	

The enhancement of collagen cross-linkages has been a subject of much recent research over the years and predominantly focuses on the use of chemical agents. Aldehydes have an extensive and long tradition as chemical cross-linkers, with glutaraldehyde and formaldehyde being the most widely exploited compounds [Barbani *et al.*, 1995; Ruderman *et al.*, 1973]. Alternative chemical compounds used also include: (1) hexamethylene diisocyanate and polyepoxy compounds which form cross-links by becoming part of the actual linkage [Zeeman et al., 1999; Chvapil *et al.*, 1993; Tu *et al.*, 1993a and 1993b], (2) acyl azide and carbodiimides [Duan and Sheardown, 2005; Petite *et al.*, 1990; Nimni *et al.*, 1988b] offer alternatives by mediating amide bonds between the free carboxylic and amino groups which are present in collagen molecules, (3) chromium tanning which is frequently exploited by the leather industry [Bradley and Wilkes, 1977]. However, the primary concern is that residual cross-linking agents or, the leaching/compound released during degradation can cause local incompatibility, inflammation, immunological responses and, ultimately, implant rejection [Olmo *et al.*, 1996; Qutiesh and Dolby, 1991; Meade and Silver, 1990].

1.3. TRANSGLUTAMINASES: PROTEIN CROSS-LINKING ENZYMES

The term transglutaminase was first introduced by Clarke and co-workers in 1957 to describe the transamidating activity of an enzyme found within the tissue of guinea pig livers [Clarke *et al.*, 1957]. Since their initial discovery, many types of transglutaminases have been discovered. Many but not all mediate a Ca^{2+}-dependent acyl transfer reaction between the γ-carboxamide group of a peptide-bound glutamine residue and the ε-amino group of a peptide-bound lysine [Lorand and Conrad, 1984]. The resulting covalent isopeptide bonds (with stable double-bond character) were first demonstrated by Pisano and co-workers in the stabilisation of fibrin monomers as being resistant to chemical degradation and most proteases [Pisano *et al.*, 1968]. The specificity of the TG for peptide-bound glutamine residues distinguishes them from similar enzymes involved in glutamine metabolism. The Enzyme Commission has classified them as EC 2.3.2.13 and recommends the denomination "R-glutaminyl-peptide-amine-γ-glutamyl transferase", although the commonly used denomination remains "TG" [Lorand and Conrad, 1984].

1.3.1. Mechanism of the cross-linking reaction

The TGs catalyse the post-translational modifications of proteins, in a process that results in the formation of polymerised cross-linked proteins [Aeschlimann and Paulsson, 1994; Lorand and Conrad, 1984]. Additionally, the TGs catalyse a number of distinct reactions that lead to post-translational modification of a specific glutamine residue in the substrate [Folk and Finlayson, 1977]. This allows the addition of new properties to the protein substrates, thereby leading to an enhancement of the substrate function, or more generally, by altering it. The TG-mediated post-translational modifications of proteins can lead to the cross-linking of two proteins through the catalysis of an acyl transfer reaction that results in the formation of a covalent ε(γ-glutamyl)lysine linkage between the proteins, whereby the acyl donor is a peptide-bound glutamine residue, while a peptide-bound lysine acts as the acyl acceptor [Folk and Chung, 1973]. Nucleophilic attack directed by the active site thiol group of the enzyme to the γ-carboxamide group of the glutamine residue in the substrate protein leads initially to the formation of the γ-glutamylthiolester intermediate, with the associated release of ammonia [Pedersen *et al.,* 1994]. The reaction then further proceeds, driven by the release of ammonia and its subsequent protonation, readily under physiological conditions. Upon formation of a γ-glutamylthiolester intermediate, the second amine donor substrate binds to the acyl-enzyme complex and attacks the thiolester bond. The acyl group is thus transferred to the acyl receptor substrate which results in the formation of an isopeptide bond and resulting in the release of the enzyme. At this stage, the active site, Cys^{277}, is re-established to its original form (in most eukaryotic TGs, Ca^{2+} is initially required to induce the necessary conformational change), thereby able to participate in further catalysis turnover cycles. The covalent acyl-enzyme intermediate is believed to be the rate-limiting step in this reaction. This reaction is commonly known as the "cross-linking" reaction and shown in Figure 1.3.

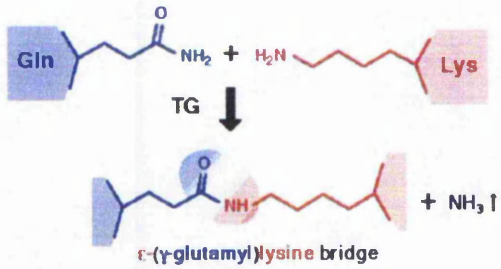

Figure 1.3. The formation of a covalent ε(γ-glutamyl)lysine linkage, commonly referred to as the "cross-linking reaction" for transglutaminases. In this reaction, peptide-bound glutamine residues act as acyl donors and a peptide-bound lysine as the acyl acceptor [Figure drawn using Adobe Photoshop®]

It has been shown that the TGs are more selective towards peptide-bound glutamine substrates than compared to the amine donor lysine residue [Aeschlimann and Paulsson, 1994], which probably defines and determines their physiological function [Gorman and Folk, 1984]. However, residues preceding accessible amine donor lysine in a native protein may exert their full influence on the enzyme's cross-linking potential as they might have an effect on the tertiary conformation of the protein and, consequently, may hinder the accessibility of the enzyme [Grootjans *et al.*, 1995]. Therefore, conformational variations between the different TGs often can result in different affinity/specificity of these enzymes for certain glutaminyl substrates [Aeschlimann and Paulsson, 1994]. The covalent cross-links resulting from TG activity are extremely stable structures which were thought to be irreversible in most situations and resistant to a wide range of proteases [Melino *et al.*, 1994; Griffin and Wilson, 1984; Lorand and Conrad, 1984] and has shown to be a crucial component of important biological processes including blood coagulation, differentiation of the epidermis of skin and ECM assembly [Griffin *et al.*, 2002]. However, it has now been shown that the hydrolysis of the ε(γ-glutamyl)lysine isopeptide by cytosolic TG and Factor FXIIIA is possible under certain appropriate conditions [Parameswaran *et al.*, 1997]. Additionally, enzymes from secretory products of the medicinal leech have been discovered and shown to hydrolyse the ε(γ-glutamyl)lysine isopeptide bond between the γ-chains of solubilised fibrin in blood clots [Lorand *et al.*, 1996; Baskova and Nikonov, 1985].

The incorporation of primary amines in to proteins, using molecules such as histamine, putrescine, spermine and spermidine as the acyl acceptors, is a further catalytic reaction which the TGs are capable of, resulting in the formation of N'(γ-glutamyl)amine bonds [Lorand and Conrad, 1984; Birckbilchler *et al.*, 1977b; Folk and Finlayson, 1977]. A free amine on the R-group can additionally be linked to another γ-glutamyl group, on the second protein, forming an N',N'-bis(γ-glutamyl) polyamine linkage. Post-translational modification of proteins by this polyamidation step may result in altered biological activity, antigenicity and turnover rate [Aeschlimann and Paulsson, 1994]. However, in an acidic environment and when a suitable amine substrate is absent, the TGs also catalyse the hydrolysis of protein-bound glutamine to glutamic residues via a very unusual and non-ideal deamidation reaction [Aleanzi *et al.*, 2001; Folk and Finlayson, 1977; Mycek and Waelsh, 1960]. This selective deamidation of the glutamine residues has recently been proposed to act as the main factor contributing to the onset of celiac disease [Ciccocioppo *et al.*, 2003]. Furthermore, the TGs are known to be involved in the aminolysis of esters (e.g. p-nitrophenyl-acetate) by insertion of polyamine groups within the ester chain [Folk and Finlayson, 1977; Folk and Cole, 1966]. Finally, it has also been proposed that keratinocyte TG (TG1) can catalyse the ester bond formation between a specific involucrin glutamyl residue and ω-hydroxyceramides, which may be important in epidermal barrier formation by keratinocytes [Nemes *et al.*, 1999]. A summary of the reactions can be seen in Figure 1.4.

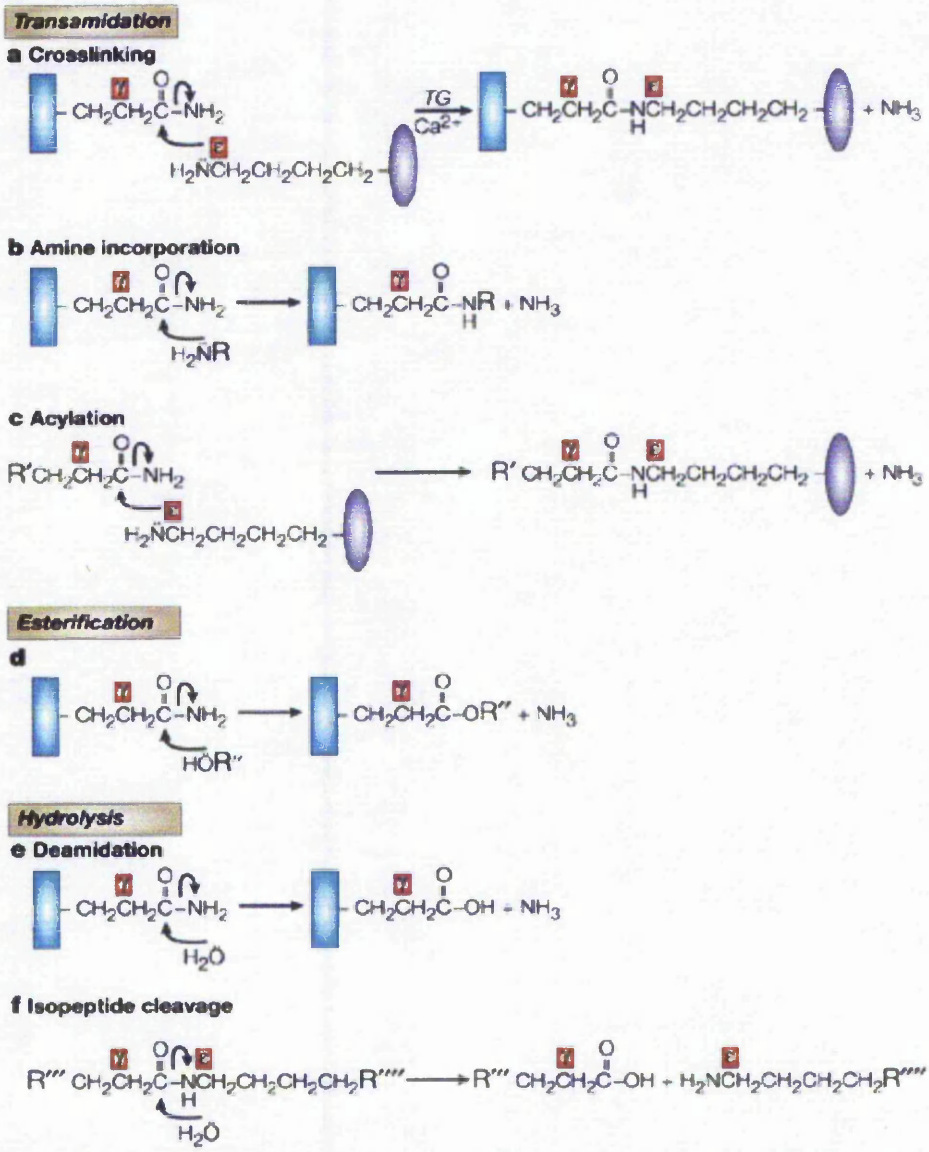

Figure 1.4. Transglutaminases catalyse various post-translational reactions. Transamidation can cause (a) protein cross-linking by forming ε(γ-glutamyl)lysine isopeptide bridge between the deprotonated lysine (Lys) donor residue of one protein (purple ellipse) and the acceptor glutamine (Gln) residue of another (blue rectangle), (b) the incorporation of amine (H_2NR) into the Gln residue of the acceptor protein (diamines and polyamines might act as a tether in a bis-glutaminyl adduct between two acceptor molecules) and (c) the acylation of a Lys side chain of the donor protein. Reactions (b) and (c) compete against the cross-linking shown in (a). The same applies to (d) esterification, but not to (e) deamidation, and (f) isopeptide cleavage [Parameswaran *et al.*, 1997]. Electron movements (curved arrows) are shown for the nucleophilic displacement reactions in the absence of enzyme. R represents the side chain in a primary amine; R', a Gln-containing peptide; R'', a ceramide; R''' and R'''' the side chains in branched isopeptides [adapted from Lorand and Graham, 2003]

1.3.2. Different forms of transglutaminase enzymes

It has been shown that TGs are expressed in a number of distantly related organisms implying a functional necessity. To date, proteins showing TG activity have been found in micro-organisms [Kanaji *et al.*, 1993; Chung, 1972; Folk and Finlayson, 1977], plants [Del Duca *et al.,* 1995; Serafini-Fracassini *et al.*, 1995], invertebrates [Singh and Mehta, 1994; Mehta *et al.,* 1990; Mehta *et al.*, 1992], amphibians [Zhang and Masui, 1997], fish [Yasueda *et al.,* 1994], birds [Puszkin and Raghuraman, 1985] and mammals [Chung, 1972]. The TGs have not only been shown to be widely distributed amongst tissues such as; epithelium, endothelium, stratum corneum, dermis, liver, spleen, bone marrow and parts of the CNS but, also, found within most physiological fluids in platelets and the lymphatic system. Furthermore, there is accumulating evidence to support the notion that several TGs can be expressed in the same tissue and perform different functions [Griffin *et al.*, 2002; Grenard *et al.*, 2001; Aeschlimann *et al.,* 1998].

As shown in Table 1.11, the mammalian TG family which are all Ca^{2+}-dependent comprises of: (a) the circulating zymogen Factor XIII which, via a thrombin-dependent proteolytic reaction, is converted to the active TG form, Factor XIIIa (plasma TG) and is involved in the wound healing process and the stabilisation of fibrin clots; (b) the keratinocyte TG isoform (TG1) which can exist as both membrane-bound or soluble forms. This enzyme is activated via several proteolytic reactions and pathways and is involved in the terminal differentiation of keratinocytes; (c) the tissue TG (TG2, tTG) which, although can be found in various tissue types in its intracellular and extracellular forms, its exact function is still debated; (d) epidermal or hair follicle TG (TG3) which also requires an additional proteolytic step prior activation and is involved in the terminal differentiation of the keratinocyte; (e) TG4 which is expressed in the prostate gland and is essential for fertility in rodents [Dubbink *et al.,* 1998]; (f) the recently characterised TG5 [Candi *et al.,* 2001]; (g) TG6 and TG7, whose tissue distribution is unknown; and (h) erythrocyte band 4.2, which is a component protein of the membrane.

Although, all the members of this family possess very similar physical attributes, the transglutaminase catalytic activity is not conserved throughout: TG2 and TG4 show inherent activity, the FXIIIA subunit, TG1 and TG3 posses some latent catalytic activity, whereas erythrocyte band 4.2, has lost its enzymatic activity completely, and only appears to serve a membrane integrity function [Lorand and Graham, 2003]. Furthermore, most members of the TGs family, apart from the microbial-sourced transglutaminases, mTG, require Ca^{2+} for activation- as it induces the necessary conformational changes of the enzyme and results in the exposure of the catalytic active site [Kashiwagi *et al.*, 2002; Chen and Mehta, 1999].

As yet, only six of the identified TGs have now been isolated, cloned and sequenced thoroughly [Aeschlimann *et al.*, 1998; Kim *et al.*, 1993; Polakowska *et al.*, 1991; Seitz *et al.*, 1991; Korsgren *et al.*, 1990; Ichinose and Davie, 1988; Ikura *et al.*, 1988). Whilst comparison of the gene products revealed a high degree of sequence homology, gene mapping has highlighted differences within the genes with distinct chromosomal localisations. Furthermore, different TG family members share similar intron and splice site distribution [Grenard *et al.*, 2001b], with TG2, TG3, TG5, and erythrocyte band 4.2, containing 13 exons, whilst the TG1 and FXIIIA subunit contain 15 exons in total. The conserved exon IX of the TG family, together with a non-homologous N-terminal extension contribute to these extra coding sequences.

There is also a certain degree of amino acid sequence homology between the different TGs. Most importantly, they share a common sequence for the active site with the amino acid sequence [Y-G-Q-C-W-V] [Gentile *et al.*, 1991; Greenberg *et al.*, 1991; Ikura *et al.*, 1988). Recently, a superfamily of archaeal, bacterial, and eukaryotic proteins homologous to mammalian TGs has also been identified using profiles generated by the PSI-BLAST program [Makarova *et al.*, 1999]. Consequently, it is apparent that the diverse biological function of the TGs that have so far been identified, are due to the differences in their primary structure and their requirement of widely differing optimal environments/conditions. To date, well characterised TG functions are those relating to FXIIIA subunit and TG1 [Aeschlimann and Paulsson, 1994].

Table 1.11: Transglutaminase nomenclature [adapted from Lorand and Graham, 2003; Griffin *et al.*, 2002]

Protein	Gene name	Alternative name	Tissue expression	Residues (Mw, kDa)	Activity and regulation	Localisation (and function)
FXIIIA subunit	F13A1	Fibrin-stabilising factor, Laki-Lorand factor, pro-fibrinoligase, plasma pro-TG	Platelets, astrocytes, dermal dendritic cells, chondrocytes, placenta, plasma, synovial fluid	732 (83)	Latent (thrombin activated); Ca^{2+} activated, reducing agent required	Cytosolic, extracellular; (blood coagulation, bone growth, wound healing, ECM stabilisation)
TG1	TGM1	TG_K, keratinocyte TG, particulate TG, membrane-bound TG, TG-B	Keratinocytes, brain	814 (90)	Latent (protease activated); Ca^{2+} activated, reducing agent required	Membrane, cytosolic; (cell-envelope formation in the differentiation of keratinocytes)
TG2	TGM2	TGc, tissue TG, liver TG, endothelial TG, erythrocyte TG, G_h, $G_{hα}$, tTG,	Ubiquitous	686 (80)	Yes; Ca^{2+} activated, reducing agent required	Cytosolic, nuclear, membrane, cell surface, extracellular ; (cell death, cell differentiation, matrix stabilisation, adhesion protein, cell signalling)
TG3	TGM3	TG_E, epidermal TG, callus TG, hair follicle TG, bovine snout TG	Squamous epithelium, brain	692 (77)	Latent (protease activated); Ca^{2+} activated, reducing agent required	Cytosolic; (cell-envelope formation during terminal differentiation of kertainocytes, hair shaft formation)
TG4	TGM4	TG_P, prostate TG, vesiculase, dorsal prostate protein 1 (DP1), major androgen-regulated prostate secretory protein	Prostate	683 (77)	Yes; Ca^{2+} activated, reducing agent required	Unknown; (semen coagulation)
TG5	TGM5	TG_X	Ubiquitous except for the CNS and lymphatic system	719 (81)	Yes; Ca^{2+} activated	Unknown; (epidermal differentiation)
TG6	TGM6	TG_Y	Unknown	706 (79)	Yes (?)	Unknown; (unknown)
TG7	TGM7	TG_Z	Ubiquitous	710 (80)	Yes (?)	Unknown; (unknown)
Band 4.2	EPB42	B4.2, ATP-binding erythrocyte membrane protein band 4.2	Red blood cells, bone marrow, foetal liver and spleen	691 (77)	No enzymatic activity	Membrane; (structural protein, membrane skeletal component)
Bacillus TG	Bacterial TG, mTG			245 (28)	σ factor regulated	Spore coat formation
Streptomyces TG				331 (37)	Proteolytically activated	Unknown

1.3.2.1. Erythrocyte band 4.2 protein

Erythrocyte band 4.2 (pallidin) is a 77kDa protein, encoded by a 20kb gene and is a major structural component of the cytoskeletal network underlying the red blood cell membrane- often expressed in high levels in erythroid cells [Cohen *et al.*, 1993; Korsgren *et al.*, 1990]. Erythrocyte band 4.2 is the only catalytically-inactive member of the TG family as it features an alanine residue instead of cysteine in the active site and, as such, is believed to only perform a structural role [Aeschlimann and Paulsson, 1994; Korsgren *et al.*, 1990].

1.3.2.2. Transglutaminase X, Y and Z

Recently, three additional members of the transglutaminase family have been discovered; transglutaminases 5 (TG_X; TGx), 6 (TG_Y; TGy), and 7 (TG_Z; TGz) [Griffin *et al.*, 2002]. Although, their structure, gene localisation and organisation and mRNA expression patterns have already been revealed, their full physiological functions have not yet been fully characterised [Griffin *et al.*, 2002; Grenard *et al.*, 2001; Aeschlimann *et al.*, 1998].

1.3.2.3. Prostate transglutaminase

Prostate transglutaminase (TG4) was first isolated and purified from the dorsal prostate and coagulation glands of rats [Romijn, 1990; Seitz *et al.*, 1990; Wilson and French, 1980]. In contrast to other types of TGs, little is known about the full physiological function of TG4. Although not fully characterised, the prostate TG has been shown to be a 150kDa homodimer consisting of two highly glycosylated and acetylated polypeptide chains that possess a lipid anchor [Wilson and French, 1980]. It has been proposed that TG4 may play a role in suppressing the immune response elicited by immuno-competent cells in the female tract against sperm cells [Paonessa *et al.*, 1984; Mukherjee *et al.*, 1983].

1.3.2.4. Epidermal TG

TG3 was initially isolated and purified, in 1975, by Buxman and Wuepper from human callus, bovine stratum corneum and snout epidermis [Ogawa and

Goldsmith, 1976; Buxman and Wuepper, 1975]. TG3 is believed to be involved in the formation, via cross-linking, of the cornified envelope precursor proteins of the epidermis during terminal differentiation; as its activity increases as cells enter the final stage of keratinocyte differentiation: from live, nuclear to the dead, anuclear, cell envelopes [Rice and Green, 1977; Buxman and Wuepper, 1975]. Other researchers suggest that TG3 may play a role in the hair formation- through the cross-linking of the structural proteins, trichohylain and the keratin intermediate filaments, to form the rigid structural supports found within the inner root sheath cells during hair shaft formation [Lee *et al.*, 1993].

1.3.2.5. Keratinocyte TG

Keratinocyte TG (TG1) exists in keratinocytes as multiple soluble forms, either intact or proteolytically processed at the conserved sites, which themselves have varying specific activities and probably different functions [Kim *et al.*, 1995; Rice and Green, 1977]. TG1 is induced during the terminal differentiation of keratinocytes and is capable of cross-linking specific intra-cellular proteins that contribute to the formation of the cornified envelope (CE). In the granular layer of the epidermis its major function appears to be the cross-linking of keratins [Yaffe *et al.*, 1992], involucrin [Simon and Green, 1988], loricrin [Hohl, 1993], cornifin [Marvin *et al.*, 1992], filaggrin [Steinert and Marekov, 1995], cystatin-α [Takahashi and Tezuka, 1999] and elafin [Nonomura *et al.*, 1994]. Additionally, it has recently been proposed that TG1 can further catalyse the formation of ester bonds between glutaminyl residues in involucrin and ω-hydroxyceramides, which may in turn facilitate anchoring of CEs to the lipid envelope of the differentiating keratinocytes [Nemes *et al.*, 1999]. It has also been hypothesised that the cross-linking activity of intercellular TG1 may play an important role in the stabilisation of the vascular endothelium barrier [Baumgartner *et al.*, 2004].

1.3.2.6. Plasma Factor XIII

The 166kDa Factor XIIIA (FXIIIA) protein is one of the most extensively characterised members of the TG family of enzymes and its physiological role has been firmly established. FXIIIA is expressed by a wide variety of cell lines

(macrophages, megakaryocytes and monocytes) in several tissues (placenta, uterus, prostate, liver) as a homodimer of two A-subunits; each of which contain 730 amino acid residues [Carrell *et al.,* 1989; Takagi and Doolittle, 1974; Schwartz *et al.*, 1973]. When externalised into the plasma, it circulates as a 320kDa tetramer, A_2B_2, composing of two non-covalently associated catalytic A-subunits (A_2) and two non-catalytic B-subunits (B_2) [Chung *et al.*, 1974; Schwartz *et al.,* 1973]. The structure and complete amino acid sequence of the subunits of FXIIIA has been established and shown to share high homology with the other characterised acyl transferases [Carrell *et al.,* 1989; Ichinose *et al.,* 1986; Grundmann *et al.,* 1986]. But, unlike many of the other TGs, FXIIIA is a pro-enzyme which requires cleavage by the serine protease thrombin at the Arg^{37}-Gly^{38} peptide bond, prior to its activation during the final stage of the blood coagulation cascade [Lorand *et al.,* 1980; Takagi and Doolittle, 1974; Schwartz *et al.*, 1973].

FXIII is the last enzyme to be activated in the blood coagulation cascade [Lorand and Conrad, 1984]. The activation of the FXIIIa leads to the cross-linking of a number of different proteins in plasma. FXIIIa catalyses cross-linking between fibrin molecules, via the formation of intermolecular ε(γ-glutamyl)lysine cross-links between selected side chains of fibrin molecules and thus, increases the strength of blood clots [Shainoff *et al.,* 1991; Lorand and Conrad, 1984; Chen and Doolittle, 1971]; it also cross-links fibrin to fibronectin and thrombospondin to anchor the blood clot to the site of injury [Bale and Mosher, 1986; Hansen, 1984], fibrin to α_2-antiplasmin to increase the resistance of the clot to plasmin degradation [Reed *et al.,* 1991; Tamaki and Aoki, 1981] and fibrin to other coagulation substrates such as factor V, platelet actin and von Willebrand factor [Hada *et al.*, 1986]. In addition to being a critical component of the blood coagulation system, FXIIIa also cross-links ECM proteins such as fibronectin [Mosher and Schad, 1979], vitronectin [Sane *et al.,* 1988], certain collagens [Akagi *et al.*, 2002; Mosher and Schad, 1979], α-2 macroglobulin [Mortensen *et al.*, 1981], plasminogen activator inhibitor [Jensen *et al.*, 1993], myosin [Cohen *et al.*, 1981] and lipoproteins [Romanic *et al.,* 1998; Borth *et al.,* 1991].

1.3.3. Tissue transglutaminase

Tissue transglutaminase (tTG) also known as: type II TG, TG2, TG_C, G_h and $G_{h\alpha}$ is a unique member of the TG family and has been characterised in a wide variety of cells (e.g. vascular endothelial cells, smooth muscles cells of any origin, renomedullary interstitial cells, mesangial cells in the kidney and colonic pericryptal fibroblasts) and tissues (e.g. liver, lung, brain, kidney, adrenal glands, testis, pancreas, erythrocyte, macrophage, uterus and muscle) [Thomazy and Fesus, 1989; Fesus and Thomazy, 1988; Lorand and Sternberg, 1976].

1.3.3.1. Organisation and structure of the human tTG gene

The human tTG gene (TGM2) has been located on chromosome 20q11-12 and analysis of the genomic organisation of the tTG has defined it as 32.5kb long-consisting of 13 exons separated by 12 introns [Gentile *et al.,* 1994]. The tTG promoter and gene structure can be seen in Figure 1.5 and 1.6 respectively. Although only 4kb represents the exon sequences, exon 13 has been found to be the largest (accounting for almost half of the full-length cDNA) and contains the coding region of the C-terminus of the protein as well as the 3' –end of the cDNA [Fraij *et al.,* 1992]. A functional human tTG promoter contains potential responsive elements and binding sites for multiple factors that include glucocorticoid and members of the TGF-β and TNFα [Lu *et al.,* 1995].

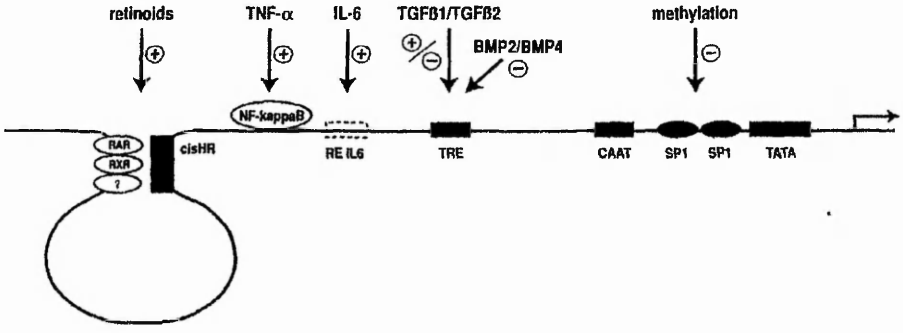

Figure 1.5. Schematic illustration of the tissue transglutaminase promoter. The TATA box and upstream SP-1 sites provide constitutive expression. Retinoids, TNFα, IL-6 and TGFβ-1 stimulate expression. TGFβ-2, BMP2/BMP4 and methylation inhibit expression [adapted from Aeschlimann and Thomazy, 2000].

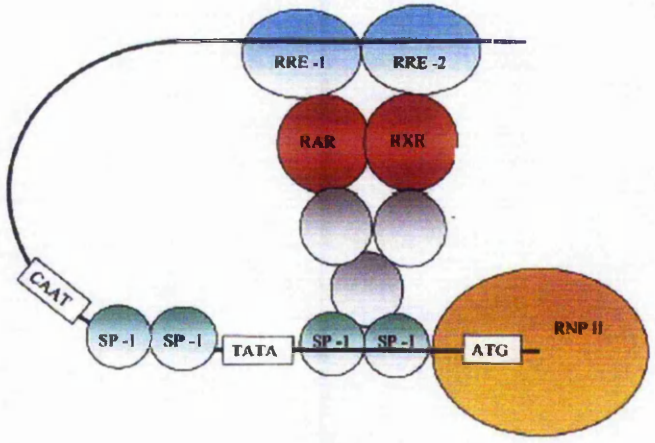

Figure 1.6. Gene structure of tTG and of its promoter. The coding region is 5'-flanked by a regulatory region of about 2kb containing regulatory elements for several transcriptional factors, including two tandem retinoid-responsive elements (RRE), which bind the retinoid receptors RAR and RXR, sites of binding for Sp1, as well as regions for regulation by TGF-β, interleukins, morphogenic protein 4 and possibly steroid receptors [adapted from Griffin *et al.*, 2002]

The complete amino acid sequence for tTG from guinea pig liver [Ikura *et al.*, 1988], bovine aorta [Nakanishi *et al.*, 1991], mouse macrophages and human endothelial cells [Gentile *et al.*, 1991] and chicken erythrocytes [Weraarchakul-Boonmark *et al.*, 1992] have been deduced from their corresponding cDNA sequences. These studies have revealed that tTG is a, highly conserved inter-species, monomeric protein consisting of 685-691 amino acids and with a molecular weight of around 77-85kDa [Aeschlimann and Paulsson, 1994]. Moreover, a 120kDa inactive form of the tTG enzyme which could be activated by proteases has also been reported in murine and human metatastic cells [Zirvi *et al.*, 1991; Knight *et al.*, 1990]. Approximately, 80% homology exists between the amino acid sequences of mouse, guinea pig and human tissue tTG- although, critically, 49 of the 51 residues in the active site region are identical. The amino acid analysis of guinea pig liver tTG has revealed that, despite having only 17 cysteine residues, the enzyme contains no disulphide bonds. Also, glycosylation does not occur, even though six potential N-linked glycosylation sites have been identified [Ikura *et al.*, 1988]. Furthermore, tTG does not possess any classical hydrophobic leader sequences and hence, its externalisation remains a mystery- although, it has been suggested that the N-terminal acetylation of the enzyme,

following initiator methionine removal may be responsible for its secretion [Muesch *et al.*, 1990; Ikura *et al.*, 1989]. Two regions rich in glutamine residues around amino acids 450 and 470 have been proposed for the calcium-binding domain in order to regulate its activity [Ichinose *et al.*, 1990].

The three-dimensional structure of tTG has been resolved based on the structures of: the latent human GDP-bound tTG dimer [Liu *et al.*, 2002], on latent sea bream liver transglutaminase [Noguchi *et al.*, 2001] and also modelled on the FXIII A-subunit due to sharing almost 75% of the active site sequence [Iismaa *et al.*, 1997]. The tTG protein consists of four distinct regions: an N-terminal β-sandwich, a α/β catalytic core and two C-terminal barrel domains (Figure 1.7). The β-sandwich domain commences with a flexible loop, followed by a short 3_{10} helix, an isolated β-strand (B_1), five anti-parallel strands (B_2-B_6) before terminating with an extra short strand close to B_1. The barrel 1 and barrel 2 domains consists of six β-sheets and a single β turn (barrel 1), or seven anti-parallel β-sheets (barrel 2). The catalytic core domain contains both, α-chains and β-sheets, in equal amounts [Iismaa *et al.*, 1997]. This organisation into the specific quadrants is highly conserved within the TG family of enzymes [Griffin *et al.*, 2002].

Studies by Iismaa and co-workers have revealed that the N-terminal domain of tTG is required for its cross-linking activity and the core domain is essential for the hydrolysis of GTP and ATP [Iismaa *et al.*, 1997]. The GTP-binding site of tTG is located in a 15-residue hydrophobic pocket between the core and barrel 1 [Liu *et al.*, 2002]. Two residues from the core (Lys^{173}, Leu^{174}) and two from the barrel [Tyr^{583}, Ser^{482}] appear to interact with the guanine base [Iismaa *et al.*, 2000]. It has also been shown that the catalytic triad (Cys^{277}, His^{335}, Asp^{358}), synonymous with the Cys-His-Asn triad found in the cysteine proteases, is located at the base of the cavity bound by the core and barrel 1 domains. The Cys^{277} in tTG is critical for the formation of the thiolester bond with a substrate bound glutamine [Lee *et al.*, 1993; Folk and Cole, 1966]. Hence, it has been concluded that the catalytic core alone is not sufficient for the TG activity; hence the flanking domains also play a role in the modulation of the transamidating activity of TG. Interestingly, the GTP-binding domain of tTG contains almost all of the conserved tryptophans of the enzyme and is thought to be responsible for the stabilisation of the transition state

[Murthy *et al.*, 2002]. It has also been shown, by the latest site-directed mutagenesis analyses, that Trp^{241} is critical for tTG cross-linking activity and is highly conserved in all of the TG members with the exception of the catalytically inactive erythrocyte band 4.2 [Murthy *et al.*, 2002].

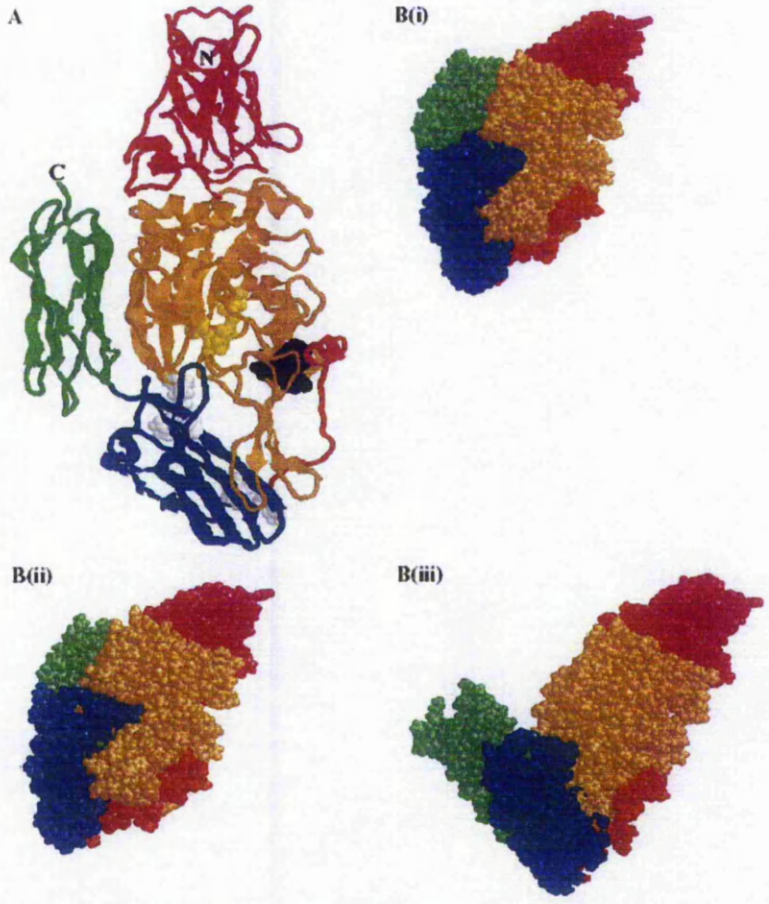

Figure 1.7. Schematic structure and ligand-dependent regulation of tTG. (A) Backbone structure of tTG. Domains I-IV are coloured respectively in magenta, orange, blue and green: the regulatory loop between domain II and III is coloured red. Amino acids involved in the active site (Cys^{277}, His^{335} and Asp^{358}), in Ca^{2+} binding (Ser^{449}, Pro^{446}, Glu^{451} and Glu^{452}) and in interaction with GTP (Ser^{171}, Lys^{173}, Arg^{478}, Val^{479} and Arg^{580}) are coloured yellow, black and light grey respectively. (B) Space-filling presentation of tTG structure in the absence of ligands (B,i) and in the presence of GTP (B,ii) and of Ca^{2+} (B,iii) to illustrate overall differences in size and in accessibility of the active site to glutamyl-substrates. The same colour is employed as for (A). the conformations in the absence of ligands (i) and in the presence of GTP (ii) are relatively similar (although interdomain interactions are strengthened in the presence of the nucleotide), while major differences are evident in the presence of Ca^{2+} (iii) [adapted from Griffin *et al.*, 2002]

Tissue transglutaminase is the only member of the family that is able to bind and hydrolyse GTP; although TG3 and TG5 can also bind to GTP but with much lower affinity. The structural domains of tTG can be seen in Figure 1.8. However, no sequence homology between tTG and any other GTP-binding proteins has been identified [Liu *et al.*, 2002; Iismaa *et al.*, 2000]. Peptide mapping and site-directed mutagenesis experiments have indicated that multiple regions interact with α_{1B}-adregenic receptor (K^{547}-I^{561}, R^{564}-D^{581} in barrel 1; Q^{633}-E^{646} in barrel 2). The phospholipase Cδ1 binding/activation site located between the receptor binding sites in barrel 2 at the C-terminal region (V^{665}-K^{672}), is specific for the GTP-bound conformation , and is crucial to the role of tTG as a GTP-binding effector protein in the transduction of α_1-adrenoreceptor signals [Hwang *et al.*, 1995]

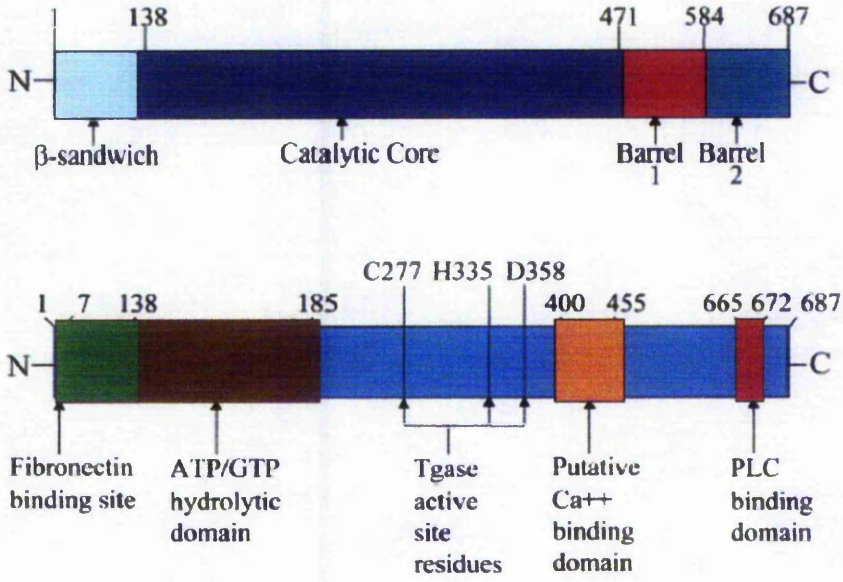

Figure 1.8. Proposed structural (top panel) and functional (bottom panel) domains of human tTG. The structural domains of tTG are based on sequence alignment with the structural domains of Factor XIII-A subunit from Yee *et al.*, 1994 [adapted from Lesort *et al.*, 2000]

Equilibrium dialysis experiments have suggested that up to six calcium ions are capable of binding simultaneously to the tTG enzyme. However, crystallographic studies of calcium-bound FXIIIa indicate a single calcium binding site. The structure of the Ca^{2+}-bound form of tTG remains unresolved, whereas the putative

Ca^{2+}-binding site is distorted in the tTG-GDP structure by the bound nucleotide [Liu *et al.*, 2002]. Two regions rich in glutamine residues around amino acids 450 and 470, at the terminal α-helix (H_4) within the core domain, have been proposed for the calcium binding site [Ikura *et al.*, 1988].

1.3.3.2. The regulation of tTG

Many cells, such as endothelial, vascular smooth muscle, platelet and epithelial cells, express the enzyme constitutively and, often, high levels of the active enzyme are accumulated [Greenberg *et al.,* 1991; Thomazy and Fesus, 1989]. In other cells, such as monocytes and tissue macrophages, the basal expression of tTG is very low but, following exposure to an inflammatory stimulus, it is dramatically and rapidly induced [Moore *et al.,* 1984; Murtaugh *et al.,* 1984]. The expression of tTG has been shown to be regulated either by direct modification of its activity (post-translational regulation) or by modulation of its gene expression (transcriptional regulation) [Verderio *et al.*, 2003; Chen and Mehta, 1999; Lu *et al.*, 1995; Cocuzzi and Chung, 1986; Achyuthan and Greenberg, 1987; Folk *et al.*, 1967]. Other regulators of tTG activity include phospholipids and NO donors [Catani *et al.,* 1998; Lai *et al.*, 1997; Fesus *et al.*, 1983]. Lai and co-workers demonstrated that the membrane lipid, sphingosylphosphocholine, can serve as specific cofactor that causes a reduction in the Ca^{2+} requirement for the activation of the enzyme [Lai *et al.*, 1997].

1.3.3.3. Localisation and cellular distribution of tTG

In short, although tTG has been seen, predominantly, as a cytosolic protein (80% of total enzyme), increasing evidence suggests that 10-15% is localised at the plasma membrane. Furthermore, recent studies have indicated the presence of tTG antigen and activity within the ECM. So far numerous cell-surface, intracellular, intranuclear, and extracellular tTG substrates have been identified which also potentially pinpoint the enzyme's localisation and are summarised in Table 1.12.

Table 1.12: Known mammalian tTG substrates [adapted from Csosz *et al.*, 2002; Griffin *et al.*, 2002]

ECM and cell surface	Blood plasma	Cytosol	Organelle Proteins	Nucleus	Others
β-casein	α_2-macroglobulin	Actin	α-oxoglutarate dehydrogenase	Core histones	Substance P
Collagen	α_2-plasmin inhibitor	Aldolase A	Acetycholine esterase	Importin-α3	Phospholipidase A_2
Erythrocyte membrane	C1-inhibitor	β -crystallin	CD38	pRB	Midkine
Fibronectin	Fibrin/fibrinogen	β -tubulin	Cytochromes		Wheat gliadin
IGFBP-1	Fibronectin	C-CAM	Erythrocyte band III		Whey proteins
Involucrin		Crystallins	Histone H2B		Soy Protein
Laminin		GADPH			Pea legumin
LTBP-1		GST			Candida albicans surface proteins
Loricrin		Glucagon			HIV envelope glycoproteins gp120 and gp41
Nidogen		Lipocortin I			HIV aspartyl proteinase
Osteocalcin C1-inhibitor		Melittin			Hepatitis C virus core protein
Osteonectin		Myosin			
Osteopontin		Phosphorylase kinase			
Protein band 3		PLA_2			
Spectrin		RhoA			
Vitronectin		Secretory vesicle IV			
		Substance P			
		Tau protein			
		Thymosin β			
		Troponin T			

Although the widespread organ distribution of tTG is attributed to its constitutive expression in endothelium, smooth muscle, fibroblasts and many other organ-specific cell lines such as stromal cells of the cycling endometrium and Z lines of sub-pericardial/ubendocardial myocytes [Thomazy and Fesus, 1989], tTG has long been described as a cytosolic protein since the majority of the enzyme is found in the cell cytoplasm where it could interact with cell membranes, cytoplasmic and cytoskeletal proteins. Since tTG does not posses any classical hydrophobic leader sequence and shows cytoplasmic characteristics, its presence in the cell membrane fraction is of little surprise [Juprelle-Soret *et al.,* 1988; Tyrrell *et al.,* 1986; Slife *et al.*, 1985; Griffin *et al.*, 1978]; although accumulating evidence suggests that the transport of tTG to the cell surface may involve non-covalent association with $\beta_1$and β_3 integrins during biosynthesis [Akimov *et al.*, 2000].

1.3.3.3.1. Cytosolic tTG

Many cytosolic proteins have been described *in vitro* as potential glutamine substrates for TGs, only a few have actually been verified as physiological substrates of tTG. Amongst those recognised substrates are: RhoA [Singh *et al.,* 2003], GAPDH- GST [Ikura *et al.,* 1998], β-tubulin [Lesort *et al.,* 1998], thymosin β_4 [Huff *et al.,* 1999], vimentin [Clement *et al.,* 1998], c-CAM [Hunter *et al.*, 1998], troponin T and myosin [Harsfalvi *et al.*, 1991], actin, β-crystallin, lipocortin I, PLA_2, glucagons, melittin and secretory vesicle IV [Aeschlimann and Paulson, 1994; Cohen *et al.*, 1985]. It has been proposed, due to the ability of tTG to cross-link these cytoskeletal proteins that, the enzyme may also be able to bind these proteins or, be present within the cellular fraction by virtue of the catalytic self-imposed cross-linking to stress fibres which, as a consequence, defines its distribution in the sub-cellular region [Chowdhury *et al.,* 1997].

1.3.3.3.2. Nuclear tTG

Tissue transglutaminase has recently been identified in the nucleus, both as a cross-linking enzyme and, also, as a G-protein [Peng *et al.,* 1999; Feng *et al.*, 1996; Lesort *et al.,* 1998; Singh *et al.,* 1995]. Sub-cellular fractionation studies of human neuroblastoma SH-SY5Y cells demonstrated that 7% of total tTG was

localised in the nucleus, of which only 6% was co-purified with the chromatin associated proteins and the remaining 1% located in the nuclear matrix fraction [Lesort *et al.*, 1998]. The same authors report that the basal activity of the tTG found within the nuclear fraction is comparable to that within the cytosol. Furthermore, the nuclear tTG can be activated, *in situ,* by the endogenous calcium-mobilising agent maltotoxin [Lesort *et al.*, 1998]. The high molecular weight of tTG (78kDa) does not allow passive diffusion nor binding to importin-α3.

To date, several potential nuclear substrates for tTG have been identified including histones [Ballestar *et al.,* 1996; Shimizu *et al.*, 1997], the retinoblastoma protein [Oliverio *et al.,* 1997] and transcription factor SP1 [Han and Park, 2000]. It has been suggested that tTG may play a part in the contribution of cell cycle progression [Mian *et al.,* 1995] and during apoptosis- the programmed cell death mechanism- by modification of nuclear substrates by the TG-catalytic polyamination or cross-linking reactions. In cells undergoing apoptosis, tTG-mediated polymerisation of the retinoblastoma gene product (pRB), a pivotal player in the control of G_1/S phase transitions, may be a key signal for the initiation of apoptosis [Oliverio *et al.*, 1997]. On the other hand, tTG–mediated dimerisation of H2B-H4 (core histones) may have a role in the formation of intranuclear inclusions during cell-death [Ballestar *et al.*, 1996].

1.3.3.3.3. Extracellular tTG

The extracellular environment provides a high concentration of calcium and low levels of nucleotides necessary for the activation of the TG enzyme. Many extracellular proteins are also known to serve as substrates for tTG such as: fibronectin [Martinez *et al.*, 1994; Barsigian *et al.*, 1991; Turner and Lorand, 1989], fibrin and fibrinogen [Achyuthan *et al.*, 1988], vitronectin [Sane *et al.*, 1988], osteopontin [Kaartinen *et al.*, 1997], osteonectin [Aeschlimann *et al.*, 1995], laminin-nidogen complex and β-casein [Aeschlimann and Paulsson, 1991], C1-inhibitor [Hauert *et al.*, 2000] as well as many different types of collagen [Esterre *et al.*, 1998; Kleman *et al.*, 1995; Juprelle-Soret *et al.*, 1988]. As the enzyme is such a potent cross-linker of so many ECM proteins, it has been proposed that the enzyme may play a central role in the reconstruction and/or stabilisation of

key components or, the fine structure of the ECM itself. Accumulating evidence indicates the presence of the enzyme on the cell surface and in the ECM despite the fact that the exact mechanism of secretion is still unknown: tTG lacks a hydrophobic signal peptide- a classic secretion marker for the conventional endoplasmic reticulum/Golgi–dependent mechanism [Akimov *et al.*, 2000; Verderio *et al.,* 1998; Nunes *et al.,* 1997; Aeschlimann and Paulsson, 1994; Martinez *et al.,* 1994]. Although it is known that tTG secretion requires the active-state conformation and an intact N-terminal fibronectin binding site, its uncharacteristic secretion pathway prevents it from being completely and efficiently released, although its secretion dramatically increases during tissue damage and cellular stress when it initially accumulates in the ECM in complex with fibronectin [Balklava *et al.*, 2002; Gaudry *et al.,* 1999a and 1999b; Haroon *et al.*, 1999a; Johnson *et al.*, 1999; Upchurch *et al.*, 1991].

Recently, tTG has been implicated in the storage and regulation of the extracellular pool of latent TGF-β through the incorporation of latent TGF-β binding protein (LTBP) into the ECM [Verderio *et al.,* 1999; Taipale *et al.,* 1994]. Since the storage and subsequent release of the ECM-bound latent TGF-β is recognised as a key mechanism is the ECM remodelling and wound healing and development [Border and Ruoslahti, 1992; Massague, 1990; Nakajima *et al.*, 1997], the regulation of the extracellular pool of tTG may therefore be a key factor in localising and concentrating TGFβ complexes before activation during the tissue modelling [Verderio *et al.*, 1999].

1.3.3.4. The tTG-fibronectin association

Among the extensive list of tTG-substrate interaction, the best characterised protein is the association of tTG with fibronectin (FN). Although initial studies by Fesus and co-workers first identified four tTG sensitive glutamine residues on human plasma FN [Fesus *et al.,* 1986], only subsequent analyses of proteolytic FN fragments confirmed that tTG binds to FN, via a non-covalent attachment, involving the first seven amino acids of the N-terminal end of the enzyme, to form a complex which implicated FN as a specific carrier for tTG [Achyuthan *et al.,*

1995; Jeong *et al.,* 1995; Lorand *et al.*, 1988]. The first *in situ* confirmation of a tTG-FN complex was provided by Martinez and co-workers who established that endothelial cell-surface tTG immobilised and processed FN at the basolateral surface of the endothelial cell in a transamidase-independent manner [Martinez *et al.*, 1994]. tTG co-localisation with FN in the pericellular matrix has since been confirmed by confocal and immuno-gold electron microscopy [Gaudry *et al.*, 1999a]. Rotary shadowing electron microscopy studies of guinea pig liver tTG-FN complexes showed that the enzyme binds within 5 to 10nm of the N-terminus of the thin FN strands and with a stoichiometry of 2:1 [LeMosy *et al.,* 1992]. This interaction is thought to be mediated by a 42kDa gelatin-binding fragment of FN that lacks any known integrin binding motifs [Radek *et al.*, 1993; Turner and Lorand, 1989]. Further investigations to define the functionality of cell-surface tTG and the tTG-FN complex, exploited antisense RNA disruption of tTG expression and concluded an impairment of cell spreading and adhesion [Jones *et al.*, 1997]. Contrastingly, *in vitro* cell adhesion experiments indicated that the cell-surface tTG serves to promote cell adhesion and spreading [Isobe *et al.*, 1999]. Interaction of tTG with multiple integrins of the β_1 and β_3 subfamilies, particularly the $\alpha_5\beta_1$ integrin, suggests that tTG may be an integrin adhesion co-receptor for FN that promotes TGFβ-mediated FN assembly [Akimov and Belkin, 2001; Gaudry *et al.,* 2000; Akimov *et al.*, 2000] while other data suggests that its binding to FN mediates adhesion to heparan sulphate proteoglycan receptors such as the syndecans [Verderio *et al.*, 2003].

1.3.3.5. The proposed roles of tTG

Although tTG was the first and most extensively studied member of the TG family, its precise physiological functions still remains elusive [Clarke *et al.,* 1957]. Emerging evidence suggests that tTG may have roles in many diverse biological functions including cell signalling [Feng *et al.*, 1996; Hwang *et al.*, 1995; Nakaoka *et al.*, 1994; Im and Graham, 1990], receptor mediated endocytosis/phagocytosis [Murtaugh *et al.*, 1983; Schroff *et al.*, 1981], cell activation and differentiation [Aeschlimann *et al.*, 1993; Gentile *et al.*, 1992; Birckbichler *et al.*, 1978; Birckbichler and Patterson, 1978], apoptosis and necrosis [Fesus *et al.*, 1987 and 1989; Lim *et al.*, 1998; Nemes *et al.*, 1996; Knight *et al.*, 1991], insulin secretion

[Bungay *et al.*, 1986; Sener *et al.*, 1985]; inflammation [Upchurch *et al.*, 1991; Bowness *et al.*, 1994; Valenzuela *et al.*, 1992; Weinberg *et al.*, 1991], fibrosis [Johnson *et al.*, 1997; Mirza *et al.*, 1997; Griffin *et al.*, 1979], neurodegenerative diseases [Gentile *et al.*, 1998; Johnson *et al.*, 1997; Kahlem *et al.*, 1996], celiac disease [Molberg *et al.*, 1998] and tumour growth and metastasis [Knight *et al.*, 1990; Mehta *et al.*, 1990; Hand *et al.*, 1987].

Over the years, evidence has begun to accumulate rapidly concerning the interaction of cell-surface tTG, cells and the ECM. The pioneering studies have shown that TG-mediated cross-linking of ECM proteins promotes cell adhesion [Martinez *et al.*, 1994; Sane *et al.*, 1991; Fesus *et al.*, 1986] and that, the induction/overexpression of tTG in several cultured cell lines leads to an enhancement of cell adhesion and reduced susceptibility to detachment by trypsin [Gentile *et al.*, 1992; Cai *et al.*, 1991]. Equally, the reduced expression of the enzyme (antisense RNA) or, its inactivation with cell-surface targeted antibodies, has also demonstrated that a decrease in cell adhesion and spreading occurs [Verderio *et al.*, 1998; Jones *et al.*, 1997]. The interaction between cells and the ECM is important in a wide range of biological phenomena, which includes cell proliferation, suppression of apoptosis, cell migration, regulation of gene expression, differentiation, blood clotting and wound healing [Critchley *et al.*, 1999], as detailed in Figures 1.9 and 1.10.

This interaction between the cells and the ECM is achieved through the action of specific cell surface receptors, namely the integrins. The integrins are heterodimeric proteins composing of an α- and β- subunit, each of which has a large extracellular domain and, a shorter, cytoplasmic domain and, at least 18 distinct α and 8 β subunits are known to exist in vertebrates [Petruzzelli *et al.*, 1999]. The association of the α-subunit (120-180kDa), β-subunit (90-110 kDa), along with the presence of divalent cations are essential for ligand binding [Gailit and Ruoslahti, 1988]. The majority of integrins, following activation, are able bind to a wide range of ECM proteins, such as fibronectin, vitronectin, fibrinogen, laminin, collagen and the complement component, iC3b [Etzioni, 1999; Detmers *et al.*, 1987]. Activation is normally initiated by "inside-out" signalling pathways- in which the integrins undergo dynamic changes, such as the lateral mobilisation of

their subunits and pro-conformational changes within the domains, resulting in the alteration in the affinity of the integrin or, clustering of the heterodimers into multimers [Sims *et al.*, 1991; Detmers *et al.*, 1987]. Once bound to the desired physiological ligand, the integrins become clustered at focal contacts and transduce signals to the cell, via "outside-in" signalling pathway, by associating with adapter proteins though the β-cytoplasmic tails [Giancotti and Ruoslahti, 1999].

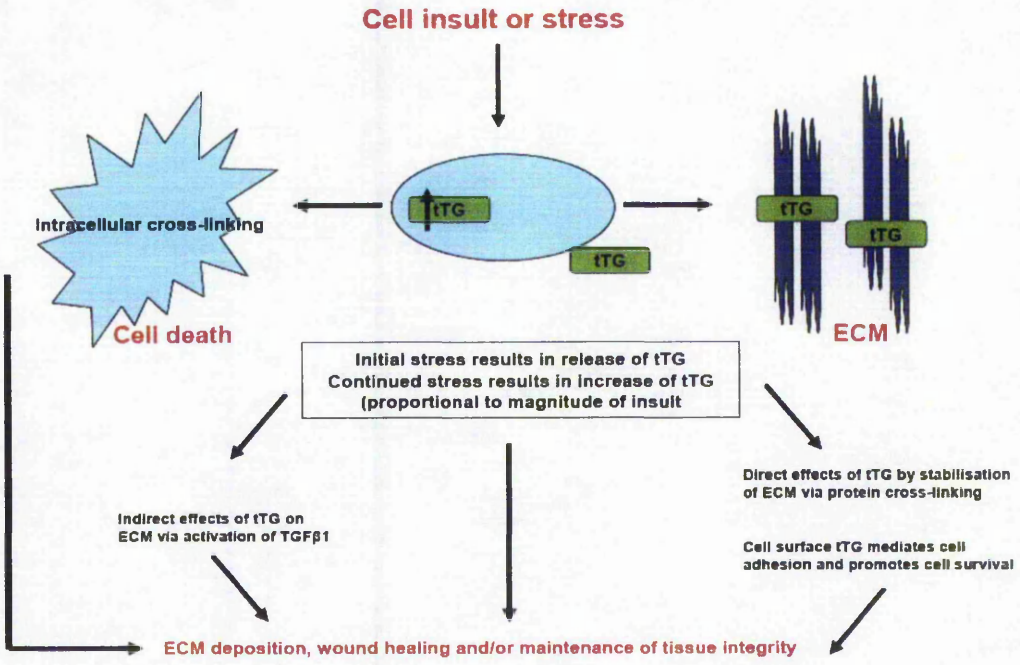

Figure 1.9. Importance of tTG in the maintenance of tissue integrity following cell stress/injury. tTG is normally secreted into the ECM in relatively low amounts. Following insult or stress, up-regulation or leakage of tTG often occurs, resulting in further enzyme externalised into the matrix and the accompanied by the massive intracellular cross-linking of the tTG-containing dying cells following loss of Ca^{2+} homeostasis. Abbreviations: tTG, tissue transglutaminase; ECM, extracellular matrix [adapted from Griffin *et al.*, 2002]

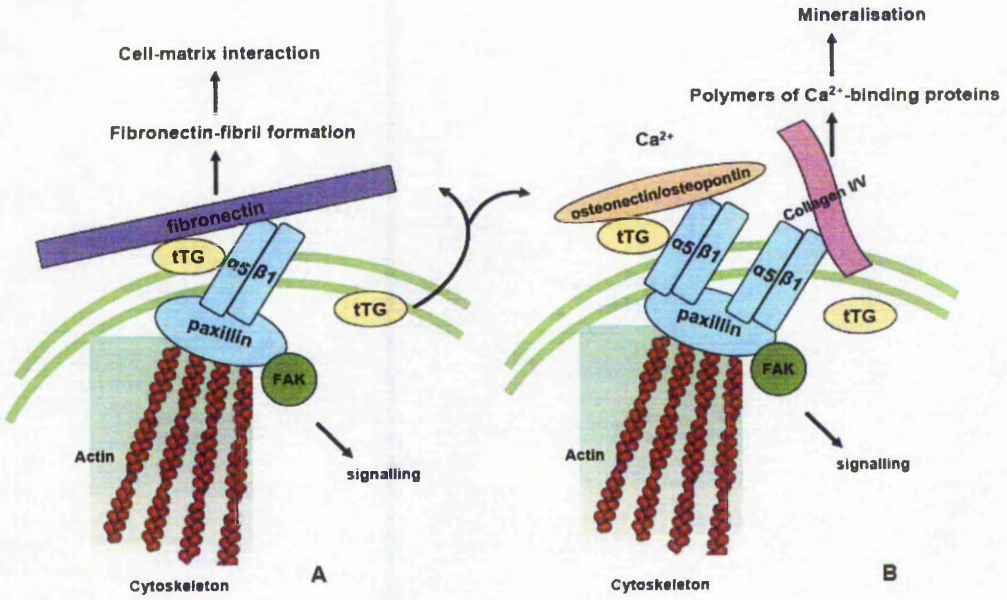

Figure 1.10. Tissue transglutaminase mediates cell-matrix interactions and also promotes mineralisation. tTG acts as an integrin co-receptor and binds very tightly to fibronectin, thereby aiding the organisation of the ECM. Through interactions with adhesion components such as paxillin and FAK, the $\alpha_5\beta_1$ integrin receptor can influence intracellular signalling and the cytoskeleton. (A) the plastic (reversible) processes of cell-matrix interactions are dependent only on non-covalent associations with tTG (B) irreversible mineralisation requires the covalent cross-linking of connective-tissue substrates (osteonectin/osteopontin and collagens) by the Ca^{2+}-activated enzyme. Unlike in the mineralisation process, the enzymatic activity of tTG is not required for cell-matrix interactions. FAK, focal adhesion kinase [adapted from Lorand and Graham, 2003]

However, not all integrins are always reactive with the ligand molecules and, hence, various modulating molecules have been proposed [Isobe *et al.*, 1999]. These investigators demonstrated that, *in vitro,* cell adhesion mediated by tTG was not dependent on the cross-linking ability but, instead, was found to be dependent via association with the $\alpha_4\beta_1$ integrin. Tissue transglutaminase when bound to FN and through cross-linking with the syndecan receptors has been shown to induce outside-in signal transduction resulting in the activation of protein kinase Cα (PKCα) [Verderio *et al.*, 2003]. Immuno-histochemistry and electron microscopy evidence has also indicated that in cells, undergoing attachment and spreading, the presence of the enzyme was found to be concentrated at adhesion points which were rich in β_1 integrin. It was also proposed that these areas could also serve as initial focal points of enzyme externalisation [Gaudry *et al.*, 1999a and 1999b].

As described, the overexpression of tTG increases its amount on the cell surface and also enhances cell adhesion and spreading [Verderio *et al.,* 1998]. Hence, the presence of integrin-bound tTG complexes on the cell surface suggests a novel mechanism (the enzyme participates in the RGD-dependent cell adhesion process via complexation with FN, i.e. tTG acts as a co-receptor) which could potentially induce the changes in adhesion and spreading characteristics [Akimov and Belkin, 2001; Akimov *et al.,* 2000]. However, a recent study demonstrated that both artificial and physiological matrices of tTG-FN complexes also play a distinct adhesive role [Verderio *et al.*, 2003]. Furthermore, findings reported by Fogerty and Mosher, demonstrated that cells incubated with antibodies directed against the α_1 and β_5 integrins showed a reduction in the attachment and migration characteristics [Fogerty and Mosher, 1990], the ability of an anti-tTG MAb to reduce (in a dose-dependent manner) the cell attachment and migration characteristics of a wide variety of other cells lines, highlights the importance of tTG in the migration of cells [Balklava *et al.*, 2002; Heath *et al.*, 2001; Verderio *et al.*, 1998; Jones *et al.*, 1997]. Collectively, these *in vitro* models are unlikely to represent the complexity of cell migration *in vivo*. However, initial *in vivo* cell migration studies on punch biopsy-induced skin lesions appear to show a significant delay in wound closure in tTG-deficient mice compared to control wild type counterparts [Mearns *et al.*, 2002; Nanda *et al.*, 2001].

Apart from the integrins, other molecules such as proteoglycans [Woods, 2001; Zimmermann and David, 1999; Woods and Couchman, 1994 and 1998] and glycosaminoglycan receptors [Bono *et al.,* 2001] are shown to play a role in cell adhesion. Although an abundant amount of literature exists; most implicating the cytoskeletal proteins (e.g. tensin, vinculin, paxillin, α-actinin, parvin/actopaxin and talin), tyrosine kinases (e.g. Src, FAK, PYK2, Csk and Abl), serine/threonine kinases (e.g. ILK, PKC and PAK), modulators of small GTPases (e.g. ASAP1, Graf, PKL and PSGAP), tyrosine phosphatases (e.g. SHP-2 and LAR PTP) and other enzymes such as PI-3 kinase and the protease calpain-II as key players involved in the focal contact mechanism, the exact biochemical pathway has yet to be elucidated [Zamir and Geiger, 2001a and 2001b].

1.3.3.5.1. Importance of tTG in wound healing

The up-regulation of tTG and the increase of tTG-mediated extracellular cross-linked products are widely associated with inflammation and the wound healing process [Aeschlimann and Thomazy, 2000]. Wound healing is a dynamic process involving a series of overlapping events that occurs in three distinct phases: inflammation, matrix remodelling and deposition, and tissue remodelling. On sustaining a tissue injury, a blood clotting cascade is activated that results in platelet aggregation and the formation of a temporary, highly cross-linked, network of fibrin and fibronectin called the provisional matrix which, ultimately, re-establishes haemostasis and maintains cell adhesion and migration [Midwood *et al.*, 2004]. Many of the extracellular roles of tTG which have implications in the early stages of wound repair involves its interaction with FN. FN participates in the regulation of the wound-repair response by providing a provisional matrix prior to collagen deposition which is essential for adhesion, migration and proliferation of cells [Davis *et al.*, 2000]. The cross-linking function of tTG in the ECM, leading to stabilisation/remodeling, has been associated with a wide range of biological processes important for tissue repair [Aeschlimann and Thomazy, 2000]. Equally, cell-surface tTG modulates and alters FN matrix stability by multimerisation following cell damage [Gross *et al.*, 2003]. Rather than through increased matrix stability tTG can also affect cell-matrix interactions either as an adhesion co-receptor of the β_1 and β_3 integrins or, as an independent cell adhesion protein [Verderio *et al.*, 2003; Balklava *et al.*, 2002; Belkin *et al.*, 2001; Akimov *et al.*, 2000; Gaudry *et al.*, 1999a and 1999b; Isobe *et al.*, 1999].

Since tTG is able to form covalent ε(γ-glutamyl)lysine cross-links, they are prime candidates for stabilising tissue during inflammation and wound healing. While it is well established that FXIII functions in cross-linking of the fibrin clot during blood coagulation and in wound healing [Barry and Mosher, 1989; Lorand and Conrad, 1984; Grinnell *et al.*, 1980], the role of tTG in this process remain inconclusive; although a potential role of tTG was proposed following the observation that $tTG^{-/-}$ knockout mice suffered delayed wound healing [Mearns *et al.*, 2002; Nanda *et al.*, 2001; De Laurenzi and Melino, 2001].

1.3.3.6. Disease states associated with tTG

In recent years, much attention has been focused on the potential role of tTG in the pathogenesis of a number of disease states. As TGs participate in such varied, cellular and extracellular processes, it is no surprise that the variations in the enzymes activity has been suggested to contribute to a wide range of pathologies when the loss of Ca^{2+} homeostasis occurs. These range from: celiac disease (CD; an enteropathy caused by the sensitivity to gliadin) patients [Arentz-Hansen *et al.,* 2000; Sollid, 2000; Molberg *et al.,* 1998; van de Wal *et al.,* 1998; Dieterich *et al.,* 1997]; tissue fibrosis [Zhang *et al.,* 2003; Grenard *et al.*, 2001; Johnson *et al.*, 1997; Mirza *et al.,* 1997; Griffin *et al.,* 1979]; Huntington's disease [Lesort *et al.,* 2000; Becher *et al.,* 1998]; Alzheimer's disease [Tucholski *et al.,* 1999; Zhang *et al.,* 1998; Murthy *et al.,* 1998; Rasmussen *et al.,* 1994]; cancer [Johnson *et al.,* 1994; Hand *et al.,* 1988; Birckbichler and Patterson, 1980]

1.3.4. Microbial transglutaminase

A transglutaminase enzyme has been isolated from the culture medium of *Streptoverticillium mobaraense* and, the first from a non-mammalian source, and is commonly known as microbial transglutaminase, mTG [Ando *et al.,* 1989]. Although the physiological role of the enzyme has still not been identified, this protein is secreted from the cytoplasm membrane, as a zymogen, and activated by proteolytic processing [Pasternack *et al.,* 1998]. Interestingly, the mTG activity is totally independent of Ca^{2+} and, in this respect, is quite different from the mammalian TGs [Aeschlimann and Paulsson, 1994; Ando *et al.,* 1989]. Following sequence analysis of this enzyme by Edman degradation, it was determined that the protein consists of 331 amino acids with a molecular mass of 37.9kDa [Kanaji *et al.,* 1993]. The amino acid sequence of mTG bears little homology to the other TGs or, in fact, to any other sequences in the current protein sequence databases; with the exception for the apparently homologous *Sv. cinnamoneum* TG [Duran *et al.,* 1998]. Only a single cysteine (Cys^{64}) has been identified as the catalytic residue in the sequence of mTG; as opposed to the active site region (consensus sequence motif of thiol proteases) possessed by the FXIII-like TGs [Kanaji *et al.,* 1993]. A recent NMR investigation demonstrated that the reaction

rate and the substrate specificity for the acyl donor of mTG are higher and lower than those of tTG (guinea pig liver) and fTG (red sea bream liver), respectively [Shimba *et al.,* 2002]. In contrast, the deamidation activity of mTG is weaker than that of fTG; implying that restrictions of the water molecule prevents it from playing a part in the role of an acyl acceptor [Ohtsuka *et al.,* 2001]. As such it has been demonstrated that mTG has a novel 3-D structure and that its catalytic mechanism is different from that of the mammalian TG enzymes

1.3.4.1. Structure of mTG

The overall crystal structure of mTG has been determined at 2.4Å resolution and can be seen in Figure 1.11 [Kashiwagi *et al.,* 2002]. The mTG molecule forms a single, and compact, disk-like domain, with overall dimensions of 65 x 59 x 41Å, and folds into a plate-like shape with a deep cleft at the edge of the molecule. The Cys^{64} residue which is essential for catalytic activity exists at the bottom of the cleft. Furthermore, the sensitivity of mTG to other cations, in the absence of a reducing agent, has been investigated and, it was shown that Cu^{2+}, Zn^{2+}, Pb^{2+} and Li^{+} were found to be strongly inhibitory to the enzyme. It is proposed that the heavy metals, such as Cu^{2+}, Zn^{2+} and Pb^{2+}, bind to the thiol group of single cysteine residue- strongly supporting the idea that this residue is part of the active site of the mTG molecule [Yokoyama *et al.,* 2004]. Microbial TG belongs to the α+β folding class, containing 11 α-helices and 8 β-strands; one of the β-sheets is surrounded by α-helices, which are clustered into three regions. The central β-sheet forms a seven-stranded anti-parallel structure and, although it is severely twisted between the β_5 and β_6 strands, there is only one hydrogen bond between the main chains of these strands (Trp^{258} and Thr^{273}). The first cluster of α-helices exists on the left side of the front view of the enzyme and is composed of α_1, α_2 and α_3 helices. The second cluster, containing the α_4, α_5 and α_{10} helices exist on the right; whereas the third cluster, composing of the α_6, α_7, α_8 and α_9 helices resides at the bottom side of the front view of mTG. The active site Cys^{64} residue is located on the loop between the α_2 and α_3 helices.

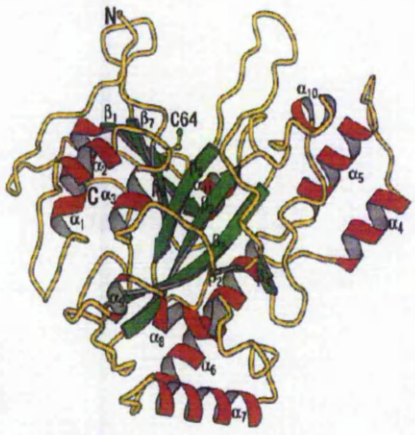

Figure 1.11. Overall structure of microbial transglutaminase (mTG): schematic ribbon drawing of the mTG as viewed from above the plate face. The secondary structure is numbered. *Ball-and-stick model*: Side chain of Cys^{64} [adapted from Yokoyama *et al.*, 2004]

1.3.4.2. Crystal structure of mTG

The crystal structures of human FXIII and red sea bream liver (fTG) transglutaminases has also been resolved [Noguchi *et al.*, 2001; Yee *et al.*, 1994], with the overall structures of fTG and FXIII resembling each other well. Figure 1.12 compares the structural properties between the active sites of mTG and fTG; their active site cysteines, Cys^{64} in mTG and Cys^{272} in fTG, both exist near the N-terminus of the α-helices (α_3 helix in mTG). In contrast to the compact, single domain structure of mTG, fTG (like human FXIII) consists of four sequential domains, named β-sandwich, core, barrel 1 and barrel 2 by Yee and co-workers [Yee *et al.*, 1994]. The active site of fTG is in the core domain which has 334 amino acid residues. Although the 3-D structure of the core domain consists of 11 α-helices and 12 β-strands and belongs to the α+β folding class, the overall folding pattern of mTG and the core domain of fTG are considerably different.

The catalytic triad of fTG consists of Cys^{272}, His^{332} and Asp^{355}- with the His^{332} and Asp^{355} residues located on the central two strands of a β-sheet. FXIII and several other cysteine proteases, such as papain [Drenth *et al.*, 1976] and actinidin [Baker and Dodson, 1980], also share a similar segment of α-helix and β-sheet that contains the catalytic triad. In contrast, the active site of mTG does not contain the cysteine protease-like catalytic triad of "Cys-His-Asp(Asn)". This anomaly is the

most striking and significant difference between mTG and the FXIII-like enzymes. Interestingly, in the mTG molecule, Asp^{255} and His^{274} occupy the positions corresponding to those of His^{332} and Asp^{355} in fTG, respectively, as seen in Figure 1.12b. Furthermore, the catalytic triad, Cys^{64}, Asp^{255} and His^{274}, of the mTG molecule superimposes well on the Cys^{272}, His^{332} and Asp^{355} triplet motif of the fTG enzyme. Thus, in the mTG molecule, the relative positions of the catalytically important His and Asp seem to be reversed relative to the Cys residue [Kashiwagi *et al.*, 2002]. In addition, the secondary structure frameworks around these residues are also very similar, implying that the two TGs are related by convergent evolution; but, however, mTG has developed a novel catalytic mechanism specialised for the cross-linking reaction. The structural differences between mTG and the FXIII-like TGs dictate the differences in their substrate specificity and reaction rate [Yokoyama *et al.*, 2004; Shimba *et al.*, 2002].

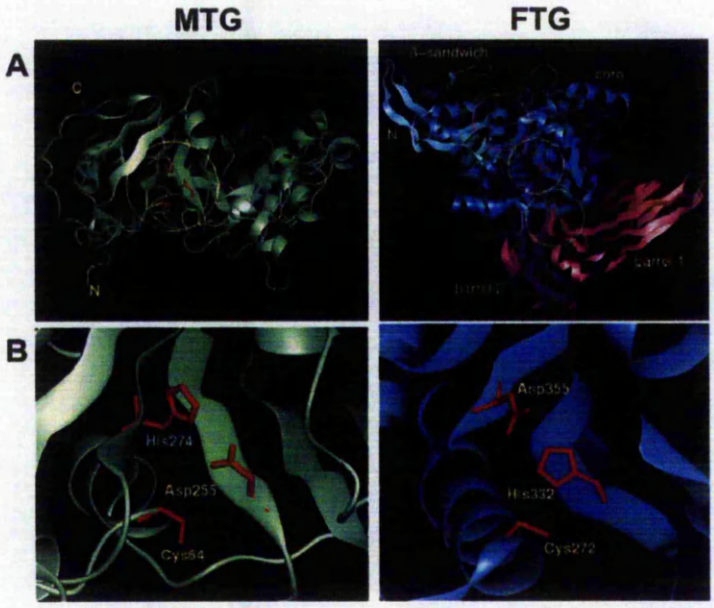

Figure 1.12. Structural comparison of mTG and fTG. A, overall structures; B, structures around the active sites of mTG (left) and fTG (right). The top views of mTG are drawn with a green ribbon model. The four domains of fTG (β-sandwich, core, barrel 1 and barrel 2 are shown in light blue, dark blue, light purple and dark purple, respectively. The catalytic triad of fTG (Cys272, His332 and Asp355) and the positionally corresponding residues of mTG (Cys64, Asp255 and His274) are represented by the red wire model. In A, the regions enclosed by yellow circles, a green circle and a purple circle represent active sites, a possible acyl donor binding site of fTG, and a possible acyl acceptor binding site of fTG, respectively [adapted from Kashiwagi *et al.*, 2002]

In short, these characteristics which include; the Ca^{2+}-independence, the higher reaction rate, the broader substrate specificity for the acyl donor, the lower activity for deamidation and the smaller molecular size are advantageous for large-scale industrial applications of the mTG enzyme. In fact, mTG is especially widely used to in the food industry to improve the physical and textural properties of many protein-rich foods such as tofu, soybean proteins, milk proteins, boiled fish paste, beef and pork sausages, chicken and fish gelatin and myosins [Nielsen, 1995; Zhu *et al.,* 1995; Nonaka *et al.,* 1992 and 1997; Seguro *et al.,* 1995a; Kang *et al.,* 1994; Kurth and Rogers, 1984; Motoki and Nio, 1983; Ikura *et al.,* 1980]. Additionally, mTG is able to incorporate amino acids or peptides into the substrate proteins, and this reaction can improve the nutritional value of food or feed proteins, because covalently incorporated amino acids or peptides behave like endogenous amino acids [Yokoyama *et al.,* 2004].

1.3.5. Biomedical applications of the TGs

The fastest-growing area of research of the TGs involves the application of these enzymes in the biomedical and biotechnology field; reflected by the increasing number of patent applications filed and the development of several medical TG-related products on the market. The earliest therapeutic application of TG was in the use of FXIII, as substitute therapy, for the treatment of blood clotting disorders [Gootenberg, 1998]. Currently, exogenous TGs have been used as biological glues in wound healing and to aid the repair of fractures and cartilage lesions [Jurgensen *et al.,* 1997]. This methodology, exploiting the recombinant form of the enzyme for large-scale use, is still being explored in surgical practice as a possible treatment for intestinal diseases [D'Argenio *et al.*, 2000]. An alternative to the direct topical application of tTG that has been considered for therapeutic use, involves the potential modulation of tTG expression via specific inducers such as the retinoids. This approach has already been utilised in the *in vivo* therapy of selected malignancies [Lentini *et al.,* 1998; Jetten *et al.,* 1990] and, also, considered as a potential strategy for the treatment of certain dermatological conditions such as acne [Bershad, 2001].

The potential to treat viral diseases has been met with considerable interest during recent times following reports on HIV infection which describe TG-mediated modifications of the viral surface glycoproteins, gp41 and gp120, which mediate HIV entry into target cells [Mariniello *et al.,* 1993a and 1993b]. In addition, it was reported that TG-mediated polyamidation brought about inhibition of HIV aspartyl-proteinase [Beninati and Mukherjee, 1992] and that the enzyme was crucial in the apoptotic clearance of infected T-lymphocytes in the establishment of HIV-associated lymphopenia [Amendola *et al.*, 1996]. It has also been documented that, in hepatitis-C-virus cellular replication, the tTG enzyme is involved in the post-translational modification of the viral core protein [Lu *et al.,* 2001]. To date, the classic exploitation of TG in biotechnology includes its diagnostic applications for autoimmune diseases, uses in the food and textile industries [reviewed in Nielsen, 1995 and Collighan *et al.,* 2002] and, more recently, as a potential biomarker, via detection of the free ε(γ-glutamyl)lysine isopeptide in body fluids, in the various diseases and disorders in which the TGs are implicated [Nemes *et al.*, 2002].

The commercial exploitation of the TGs appears at an ever increasing rate and, in some instances, in the most unusual and atypical applications, for example in the pathogenesis of infectious diseases and the development of novel vaccines for a range of bacterial and viral infections. For instance, the *E. coli* toxin Cytotoxic factor 1 acts as a TG, although in this case it maintains an absolute specificity for the GTP-binding protein Rho [Schmidt *et al.,* 1998]. In addition, the development of bacterial and yeast biofilms frequently involve TG-like modification of surface proteins [Staab *et al.,* 1999]. The most extensively exploited TG is the microbial variant and is derived from *Streptoverticillum mobaraense,* does not require Ca^{2+} for its activation [Gerber *et al.,* 1994; Pasternak *et al.,* 1998] and is abundantly available as a commercially product. This enzyme has found several applications as a biocatalyst in the food, cosmetic and textile industries [Cortez *et al.,* 2002; Kuraishi *et al.,* 1997; Bailey *et al.,* 1996; Rasmussen *et al.,* 1996; Ishii *et al.,* 1994].

1.4. PROJECT AIMS

The aim of this work is to develop a novel biomaterial which may be used for either wound dressing or, as a biological scaffold for tissue engineering in skin or bone applications. Collagen type I has been widely used in medical applications due to its distinct biological interaction within the body. It is this bioactivity that controls the growth, differentiation and behaviour of selective cells on the collagen and facilitates their organisation into functional tissues or organs. Cross-linked collagen is more suited to be used as a biomaterial for either wound dressing or as a scaffold than its native form due to the enhanced physical characteristics which includes mechanical properties, thermal stability and resistance to proteolytic breakdown. At present, collagen has been cross-linked by a variety of chemical agents as well as physical heating and UV irradiation. However, in view of the toxicity of the cross-linking agent and in-sustainability at the large-scale, it would be more useful if the collagen were to be cross-linked by a milder, more efficient and more practical means. Thus, the formation of ε(γ-glutamyl)lysine isopeptide bonds in collagen using the enzyme, transglutaminase, as a biological catalyst has considerable potential.

Therefore, the ultimate goal of this project is to use the transglutaminases to modify collagen with the view to develop novel biomaterials that are conducive to both soft and hard tissue repair and regeneration. As such the project will:

- Investigate the suitability of the transglutaminases to cross-link collagen
- Incorporate bioactive synthetic peptides/analogues into collagen using the transglutaminases
- Determine and characterise the response of cells on this new cross-linked material

CHAPTER 2:

MATERIALS AND METHODS

CHAPTER 2: MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. General chemicals

All water used was deionised using an Elgastat System 2 water purifier (ELGA Ltd., Buckinghamshire, UK) and/or Milli-Q water purifier (Millipore Waters, Watford, UK). Sterile preparation of stock solutions and chemicals were performed either by filtration through a 0.22μm Whatman sterile filter and/or autoclaving at 121°C at 1 bar for 1 hour. Most general chemicals were purchased from Sigma-Aldrich (Poole, UK) unless otherwise as stated as below:

Activa™ WM (microbial transglutaminase) was obtained from Ajinomoto Corporation Inc., Japan. Synthetic labelled peptides: FITC-TVQQEL and FITC-KKKKGY were synthesised by Alta Bioscience, University of Birmingham, UK. N,N′-methylene biascrylamide, rainbow molecular weight markers and Lowry protein assay kit were supplied by Bio-Rad Laboratories Ltd, Watford, Hertfordshire, UK. Glacial acetic acid, sulphuric acid, acrylamide, N, N′-dimethylcasein, glycine, hydrochloric acid, calcium chloride, dimethyl sulphoxide, hydrogen peroxide, sodium dodecyl sulphate, sodium acetate and sodium hydroxide were purchased from BDH (Merck), Poole, Dorset, UK. Synthetic H-Gly-Arg-Gly-Asp-Thr-Pro-OH (GRGDTP) and H-Gly-Arg-Ala-Asp-Ser-Pro-OH (GRADSP) peptides, MMP inhibitor GM6001 (Galardin; 364205) and negative control (364210) and ABTS were supplied by CalBiochem, Nottingham, UK. Synthetic peptides: EP011803 (H2N-EAQQIVPHSRNGGGRGD-COOH) and EP011804 (H2N-GKKGKKGPHSRNGGGRGD-COOH) were synthesised by Eurogentec (Thermo Bioanalysis Ltd.). Southampton, UK. Serum-free AIMV medium was purchased from GIBCO Life Technologies, Paisley, UK. FITC-labelled collagen was supplied by Merck Biosciences, Hertfordshire, UK. Biotin cadaverine and biotin-X-cadaverine were purchased from Molecular Probes, Oregon, USA. CellTiter 96®AQ One Solution Cell Proliferation assay kit was purchased from Promega, Southampton, UK. Scion Image™ software was developed by Scion Corporation, Maryland, USA.

2.1.2. Radiochemicals

[1,4-^{14}C]-Putrescine was supplied by Amersham Pharmacia Biotech, Buckinghamshire, UK. Ultima Gold™ scintillation fluid was obtained from

Packard Bioscience Ltd., Pangbourne, UK. [^{32}P]-labelled dCTP was supplied by Redivue (Amersham), GE Healthcare, Buckinghamshire, UK.

2.1.3. Immunochemicals

Anti-FAK (phospho Y397) rabbit polyclonal was purchased from Abcam, Cambridge, UK. Anti-phosphotyrosine rabbit polyclonal antibody and anti-phosphotyrosine mouse MAb were purchased from BD Transduction Laboratories, Oxford, UK. Anti-mouse IgG-HRP conjugate and Osteopontin (human colorimetric) ELISA kit were purchased from CalBiochem, Nottingham, UK. Collagen-Specific Integrins Investigators kit (ECM425) was supplied by Chemicon International, Hampshire, UK. Anti-mouse IgG-FITC conjugate, anti-mouse IgG-TRITC conjugate, anti-goat IgG-HRP conjugate and anti-rabbit IgG-HRP conjugate were purchased from Dako Ltd., High Wycombe, Buckinghamshire. CUB7402 anti-tTG MAb was supplied by Neomarkers, Fremont, USA. Anti-phospho-Akt (Ser473) rabbit polyclonal antibody and anti-Akt rabbit polyclonal antibody were purchased from New England Biolabs Ltd., Hertfordshire, UK. Rabbit IgG polyclonal antibody to mTG (from *Streptomyces mobaraensis*) was purchased from N-Zyme Biotech, Darmstadt, Germany. RayBio® Human Matrix Metalloproteinase Antibody Array I kit was supplied by RayBiotech Inc. (Insight Biotechnology Ltd.), Middlesex, UK. Anti-phospho-ERK(E4) mouse MAb, anti-phospho-JNK(G7) mouse MAb, anti-α_1 (TS2/7) mouse MAb, anti-α_2 (H-293) rabbit polyclonal antibody, anti-α_3 (H-43) rabbit polyclonal antibody, anti-α_v (P2W7) mouse MAb, anti-β_1 (M-106) rabbit polyclonal antibody, anti-β_2 (CTB104) mouse MAb, anti-β_3 (SAP) mouse MAb and anti- α tubulin (B152) mouse MAb were all purchased from Santa Cruz Biotechnology Inc., Calne, UK. Anti-phospho-FAK(Tyr397) rabbit polyclonal antibody and anti-phospho-FAK(Tyr576) rabbit polyclonal antibody were purchased from Upstate Ltd., Buckingham, UK.

2.1.4. Western blot chemicals

Rainbow-coloured molecular weight marker and enhanced chemiluminescence ECL reagent were obtained from Amersham Pharmacia, Buckinghamshire, UK. Blot absorbent filter paper and gel loading tips were supplied by Bio-Rad Laboratories Ltd, Watford, Hertfordshire, UK. Nitrocellulose membranes were purchased from Gelman Biosciences, Northampton, UK. Kodak X-OMAT

detection film was purchased from Roche Diagnostic, East Sussex, UK. LX-24 developer solution and FX-40 fixer solution were purchased from Sigma-Diagnostics, Poole, UK.

2.1.5. Northern blot chemicals

Hybond N-nylon membranes, Sephadex G50 Nick columns™ were purchased from Amersham Pharmacia, Buckinghamshire, UK. ExpressHyb™ hybridisation solution was purchased from Clontech UK Ltd., Hampshire, UK. Phoretix 1D image analysis software was developed by NonLinear Dynamics, Newcastle-upon-Tyne, UK. RNA molecular weight marker were purchased from Promega, Southampton, UK. Kodak AR/LS detection film was obtained from Roche Diagnostic, East Sussex, UK. 3MM paper wick was purchased from Whatman, Kent, UK.

2.1.6. Protein reagents

Marvel dried milk powder was purchased from domestic supply outlets.

2.1.7. Molecular biology kits and reagents

Low melting point (LMP) agarose was purchased from Bioline, London, UK. SV Total RNA isolation system, DNA and RNA standard markers and Prime-a-Gene™ system were purchased from Promega, Southampton, UK. RIPA cell lysis buffer was supplied by Santa Cruz Biotechnology Inc., Calne, UK.

2.1.8. Other consumables

Superfrost Gold coated glass and standard (uncoated and untreated) microscope slides and coverslips were purchased from BDH, Poole, Dorset, UK. Scintillation vials were supplied by Canberra-Packard, Pangborne, UK. Tissue culture, petri dishes and flasks were purchased from Corning/Bibby-Sterilin, Stone, Staffs, UK. 0.5, 1.5- and 2-ml microcentrifuge tubes, 5 ml scintillation vial inserts, 15 and 50ml sterile centrifuge tubes, 10ml sterile pipettes, automatic pipette fillers, 1ml and 200µl pipette tips, cell scrapers, 0.22µm filters and NUNC/Nalgene cryovials were all purchased from Starstedt Ltd., Leicester, UK. Tissue culture flasks T25, T7, T150, 10cm and 6cm Petri dishes, 6-, 12-, 24-, 48- and 96- well plates, improved Neubauer haemocytometer and parafilm were

purchased from Scientific Laboratory Supplies, Nottingham, UK. Whatman no.1, no.42 and 3MM chromatography/filter paper were purchased from Whatman Ltd, Maidstone, UK.

2.1.9. Equipment

Agilent Technologies 6890N Network GC system was supplied by Agilent Technologies UK Ltd., Stockport, UK. Amersham UV cross-linker was supplied by Amersham, Little Chalfont, UK. Spectrophotometer Model DU-7, centrifuges Avanti J-30 I, MSE Centaur 2, GPKR and MSE Microcentaur and Optima TLX Tabletop Ultracentrifuge were supplied by Beckman Instrument (UK) Ltd, High Wycombe, UK. Atto-minigel protein electrophoresis system was supplied by B & L Systems, Marseen, The Netherlands. Tri-Carb 300 Scintillation counter were supplied by Canberra-Packard, Pangborne, UK. Cecil 1010 spectrophotometer was supplied by Cecil Instruments Ltd., Cambridge, UK. pH meter 130 was purchased from Corning, Stone, Staffs, UK. Dionex DC-4A resin column was supplied by Dionex (UK) Ltd., Surrey, UK. Freeze Dryer (Modulyo System) was supplied by Edwards High Vacuum, Sussex, UK. MCC 340 ELISA plate reader and Gelaire BSB 4A laminar flow cabinets were obtained from Flow Laboratories, High Wycombe, UK. Water baths were supplied by Grant Instruments, Cambridge, UK. Techne Hybridser HB-1D dryer oven was supplied by Jencons-PLS, East Sussex, UK. IG150 tissue culture CO_2 incubators, laminar flow cabinet LC 2.12 and Jouan vacuum concentrator were obtained from Jouan Ltd., Derbyshire, UK. Bohlin C-VOR rheometer was supplied by Malvern Instruments, Worcestershire, UK. Soniprep 150 sonicator and Chilspin refrigerated centrifuge were supplied by MSE, Loughborough, UK. CK2 and BH2 light inverted microscopes, OM4 Ti 35mm camera and DP10 microscope digital camera were obtained from Olympus Optical Company (UK) Ltd., Middlesex, UK. Packard Liquid Tri-Carb LS scintillator was supplied by Packard Biosciences, Pangbourne, UK. Electrophoresis power supply, LKB Multiphor II semi-dry blotter and Alpha-Plus amino acid analyser were obtained from Pharmacia, Milton Keynes, UK. EZ:Faast™ amino acid testing kit was purchased from Phenomenex, Macclesfield, UK. Spectafluor 96-well ELISA plate reader and XFluor4 software were purchased from Tecan UK Ltd, Goring-on-Thames, UK. Dimension 3100 atomic force microscope was provided by Veeco Instruments Ltd., Cambridge, UK.

2.2. METHODS

2.2.1. CELL CULTURE

2.2.1.1. General cell culture

Human osteoblast (HOB) cells, isolated from explants of trabecular bone dissected from femoral heads following orthopaedic surgery as previously described [DiSilvio *et al.*, 1995] were kindly supplied by Professor S. Downes and Dr. S. Anderson (School of Biomedical Sciences, University of Nottingham) and used during this investigation. Additionally, human foreskin dermal fibroblast (HFDF) cells isolated from human neonatal foreskin were also used. Both cell lines were used during their low-passage number; ranging from between 5 to 15 passages. Cell lines were cultured and maintained, *in vitro*, as monolayers in T-flasks using DMEM, supplemented with 10% (v/v) heat-inactivated (56ºC for 1h) FCS, 1% (v/v) non-essential amino acids and 2mM L-glutamine. Periodic additions of 1% (v/v) penicillin-streptomycin antibiotic were used to avoid bacterial contamination. All cell lines were routinely cultured in a humidified-atmosphere incubator at 37°C and with 5% (v/v) CO_2 with regular medium changes every two or three days. Back-up cultures were maintained using a separate and independent supply of medium and solutions. General ATCC cell line maintenance procedures were followed at all times using aseptic and sterile techniques. All handling and manipulation of cells were confined to double HEPA-filtered laminar flow cabinets under ACGM Containment level 2 regulations. Disposal of cells and other biological wastes were autoclaved and/or disinfected as required by ACGM regulations.

2.2.1.1.1. MEF cell culture

Mouse embryonic fibroblasts (MEF) cells, isolated from tTG wild type ($tTG^{+/+}$, tTG-WT) and knockout ($tTG^{-/-}$, tTG-KO) mice, were kindly donated by Prof. Melino and Dr. De Laurenzi (IDI-IRCCS, University Tor Vergate, Rome) and also used in this investigation. Generation of these tTG-deficient mice was performed according to Melino and De Laurenzi [Melino and De Laurenzi, 2001]. Both cell lines were used during their low-passage number; ranging from between 2 to 8 passages and were cultured and maintained, *in vitro*, as monolayers in T-flasks using DMEM-F12, supplemented with 10% (v/v) heat-inactivated (56ºC for 1h) FCS, 1% (v/v) non-essential amino acids, 4mM L-glutamine, and maintained in a

humidified-atmosphere incubator at 37°C and with 5% (v/v) CO_2. Periodic additions of 0.5% (v/v) penicillin-streptomycin antibiotic were also used to avoid bacterial contamination.

2.2.1.2. Passaging of cells

Cells were routinely maintained to ensure that they were healthy and were never allowed to reach greater than 85% confluency at any one time. For routine passaging and detachment, a standard trypsinisation protocol was performed. Briefly, the cell monolayer was rinsed once with PBS (pH 7.4) prior to treatment with 0.25% (w/v) trypsin/2mM EDTA solution, in PBS (pH 7.4) at 37ºC for approximately 5 minutes. Following (visual) detachment of cells, the trypsin was inactivated by the addition of an equal volume of culture medium containing 10% (v/v) FCS. This suspension was then transferred to a centrifuge tube before being spun down at 1300rpm (300g), for 5 minutes, on a MSE bench top centrifuge. The cell pellet was then re-suspended in the appropriate volume of fresh complete medium or re-seeded at the desired cell density in the corresponding culture vessel. This was then kept in a humidified-atmosphere incubator at 37°C and with 5% (v/v) CO_2.

2.2.1.3. Cell viability, proliferation and apoptosis

2.2.1.3.1. Trypan blue exclusion assay

Cell counts and viability estimations were performed using the trypan blue exclusion technique by means of a 0.22µm sterile filtered 0.5% (w/v) trypan blue solution and a haemocytometer. An equal volume of the cell suspension and of the stain were well-mixed in a sample well by the pulsing of a pipette- this also ensured that clumps of cells were broken up to allow for a more accurate cell count. Non-viable cells stained blue due to the loss of their membrane integrity and, hence, allowed the passage of dye into the cell. Viable cells remained colourless.

2.2.1.3.2. Cell proliferation assay

Cell proliferation and viability were also measured using the CellTiter AQ One Solution Cell Proliferation™ assay kit (Promega, Southampton, UK)- a simple

colorimetric method for determining the number of viable cells. The CellTiter AQ reagent contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethyoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES). PES has enhanced chemical stability which allows it to be combined with MTS to form a stable solution. This MTS tetrazolium compound (Owen's reagent) is bioreduced by cells into a coloured formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. The assay was performed accordingly to the manufacturers' instructions. Briefly, assays were performed, in reduced lighting, simply by the addition of 20µl of CellTiter AQ reagent into the relevant samples in 100µl of culture medium. These samples were then incubated in a humidified-atmosphere incubator at 37°C and with 5% (v/v) CO_2 for between 1 to 4 hours, before the absorbance was read at 492nm using a SpectraFluor® plate reader.

2.2.1.3.3. Caspase-3 activity assay

Programmed cell death was assessed using the commercially available CaspACE™ Assay system (Promega, Southampton, UK) according to the manufacturer's instructions. Briefly, cells were seeded on the relevant substrate at a density of $2x10^5$ cells/well of a 6-well plate and allowed to proliferate overnight. The apoptotic index of the cell line was measured by the ability of caspase-3 to cleave Ac-DEVD-p-nitroaniline substrate and release a chromophore, p-nitroaniline (pNA). Apoptosis in the positive controls was induced using 1µM of the protein kinase inhibitor staurosporine, whereas the irreversible and cell-permeable pan-caspace inhibitor, Z-VAD-FMK, was added (50µM final concentration) to the negative control together with 1µM staurosporine.

2.2.1.4. Long term storage of cells

2.2.1.4.1. Cryopreservation

Prior to storage, cell cultures were maintained in an actively growing state to ensure optimum health and good recovery. A minimum concentration of 3×10^6 cells/ml were detached from the culture vessel before being resuspended in a

'freezing mixture', consisting of 10% (v/v) DMSO in FCS. This cell homogenate was then aliquotted into cryogenic storage vials and placed into an isopropanol-containing cryo-container (Mr Frosty™, Sigma, Poole, UK), before being 'stepped-frozen'- by placement into a -80°C freezer for 24 hours, after which the vials were transferred to liquid nitrogen for further long-term storage.

2.2.1.4.2. Cell revival from storage

Cells were removed from storage and placed directly into a 37°C water-bath until thawed. Immediately after thawing, the cells were transferred into a sterile centrifuge tube and 5ml of complete growth medium was added drop-wise, mixing well after each addition to ensure slow dilution. This diluted cell suspension was then centrifuged at 300g for 5 minutes to remove any of the freezing mixture. Cells were re-suspended in the appropriate cell growth medium, transferred to the appropriate tissue culture flask and incubated in a humidified-atmosphere incubator at 37°C and with 5% (v/v) CO_2 as described above (section 2.2.1.2). The medium was changed during the next 12-24 hours to ensure complete removal of residual DMSO.

2.2.1.5. Attachment and spreading assay

Cells were seeded on the relevant substrate; tissue culture plastic (TCP) and/or collagen at a density of 625 cells/mm^2 in the appropriate medium. After allowing the cells to proliferate, spread and/or attach for the required time period, the cells were gently washed once with PBS, pH 7.4. Cells were then fixed by treating them with 3.7% (w/v) paraformaldehyde in PBS, pH 7.4, for 15 minutes at room temperature before being washed twice with PBS, pH 7.4. Following fixation, cells were permeabilised by the addition of 0.1% (v/v) Triton X-100 in PBS, pH 7.4, for 15 minutes at room temperature. Samples were then, again, washed twice with PBS, pH 7.4. Visualisation of the attached or spread cells required a two-step staining procedure which stained both the cytoplasm and nucleus. Hence, following the fixation and permeabilisation steps outlined above, cells were first stained with a cytoplasm stain, 0.25% (w/v) May-Grunwald, in methanol, for 15 minutes at room temperature. This stain was then removed and the samples washed once with PBS, pH 7.4. Nuclear staining was achieved using 0.4% (w/v) Giemsa stain, in methanol (diluted 1:50 with distilled water),

which was added to the samples and incubated for 20 minutes at room temperature. Again, this stain was removed and the plates washed twice with distilled water and left to air dry.

The samples were then viewed at x400 magnification using a Nikon CK2 microscope. Three separate fixed-size, non-overlapping, random fields per sample were photographed with an Olympus DP10 digital camera and analysed using Scion Image™ software (Scion Corporation, Maryland, USA). The number of cells per image was assessed through threshold and particle analysis settings with a minimum particle size of 50 pixels. Spread cells were distinguished and characterised based upon the presence of a clear halo of cytoplasm surrounding their nucleus, following the rearrangement in the actin skeleton, as previously described by Jones and co-workers [Jones *et al.*, 1997]. These were quantified and interpreted by the density slicing and particle analysis settings available.

2.2.1.6. Cell migration: agar drop

This migration assay is based on a modification of the technique described by Akiyama and co-workers [Akiyama *et al.,* 1989]. Wells of a 6-well plate were coated with the appropriate collagen substrate or left untreated as a control sample. Sample wells were washed once with PBS, pH 7.4 and once again with bicarbonate-free DMEM buffer containing 25mM HEPES. Excess solution was removed with care to avoid damaging the collagen matrices; after which the well-plate was placed, to chill briefly, in a 4°C refrigerator. Cells were then trypsinised and centrifuged, as described earlier, before being resuspended in bicarbonate-free DMEM buffer (containing 25mM HEPES) at a concentration of $6x10^7$ cells/ml. This suspension was then mixed with a 2% (w/v) low-melting point agarose (Bioline Ltd., London, UK), maintained at just above 38°C, to achieve a final concentration of 0.2% (w/v). Immediately, 0.5µl of this agar drop was placed in the centre of each well in the well-plate. The agarose was allowed to set for 10 minutes at 4°C, before an overlay of 100µl of pre-warmed (37°C) AIM V growth medium (GIBCO Life Technologies, Paisley, UK) was added to each well; taking care not to detach the droplet nor disturb the collagen. Cells were allowed to migrate at 37°C and 5% (v/v) CO_2, in a humidified-atmosphere incubator, for the required time-period, before being fixed and stained with 0.5% (w/v) crystal violet in 70% (w/v) ethanol for 15 minutes at room temperature. Following fixation, the cells were washed twice with PBS, pH 7.4, and allowed to air dry. Samples were

then viewed at x400 magnification using a Nikon CK2 microscope and random fields per sample were photographed with an Olympus DP10 digital camera.

2.2.1.7. Alkaline phosphatase (ALP) activity

The ALP Optimized Alkaline Phosphatase EC 3.1.3.1 Colorimetric Test® kit (Sigma, Poole, UK) was used to quantify the ALP activity. Alkaline phosphatase hydrolyses p-nitrophenyl phosphate to p-nitrophenol and inorganic phosphate. The hydrolysis occurs at alkaline pH and the p-nitrophenol formed shows an absorbance maximum at 405nm. The rate of increase in absorbance at 405nm is directly proportional to ALP activity in the sample. Samples were treated according to the manufacturers' instructions. Briefly, samples and substrates were warmed up to 37°C prior use and analysis. 2.7ml of ALP Reagent A was added to 0.05ml of test sample and mixed immediately by inversion for 1 minute. 0.25ml of ALP Reagent B was then added and mixed immediately before a reading was taken against water as a reference, at 405nm, using a Beckmann DU530 UV/Vis Spectrophotometer. Further readings were taken at 1, 2 and 3 minute intervals following the initial absorbance reading. The mean absorbance change per minute was calculated and the ALP activity determined as follows:

$$\text{ALP activity (U/L)} = \frac{\Delta A/\min \times TV \times 1000}{18.45 \times LP \times SV}$$

Where:

ΔA/min = change in absorbance per minute at 405nm
TV = total volume (ml)
SV = sample volume (ml)
18.45 = millimolar absorptivity of p-nitrophenol at 405nm
LP = lightpath (1cm)
1000 = conversion factor (ml to litre)

2.2.1.8. Osteopontin (OPN) concentration levels

The Osteopontin ELISA kit (CalBiochem, Nottingham, UK) was used to quantify the concentration of osteopontin in the samples. The kit uses a polyclonal antibody to human OPN immobilised on a micro-titre plate to bind to the human OPN. Samples were treated according to the manufacturers' instructions and summarised below. The micro-titre plate was prepared by pre-washing twice with

400µl of the supplied wash buffer (PBS with additional detergents). Residual wash buffer was removed by tapping the inverted plate on lint free paper towels. 100µl of standards and the samples were added to the appropriate wells and the plate tapped gently to ensure complete covering of the coated wells. Following a 1 hour long incubation at room temperature, the contents of the wells were removed and the plate washed with 400µl of wash buffer for a total of 7 washes. After the final wash, residual wash buffer was, again, removed by tapping the inverted plate on lint free paper towels. 100µl of the labelled antibody was pipetted into each well (apart from the blank) before being sealed and incubated for 2 hours at room temperature. Following the incubation, the plate was washed as described above but, this time, the plate was washed a total of 9 times. 100µl of the substrate solution was then added to each well. The whole plate was then incubated, in the dark, for 30 minutes at room temperature. To terminate the reaction, 100µl of stop solution was then added to each well, before the absorbance was read at 450nm using a Beckmann DU530 UV/Vis Spectrophotometer; the measured absorbance being directly proportional to the concentration of human OPN in the sample.

2.2.1.9. MMP inhibition: Spreading and attachment analysis

Stock solutions of the MMP broad-range inhibitor GM6001 (Galardin; 364205) and the corresponding negative control (364210) (CalBiochem, Nottingham, UK) were prepared according to the manufacturers' instructions. Cell samples ($2x10^5$ cells/ml) were then mixed with the appropriate inhibitor before being immediately plated out on to the relevant substrate. Samples were then cultured in humidified-atmosphere incubator, at 37°C and with 5% CO_2, for the desired time points, before being subjected to the spreading and attachment analysis as described in section 2.2.1.5.

2.2.1.10. MMP inhibition: Matrix remodelling

Stock solutions of the MMP broad-range inhibitor GM6001 (Galardin; 364205) and the corresponding negative control (364210) (CalBiochem, Nottingham, UK) were prepared according to the manufacturers' instructions. Cell samples ($2x10^5$ cells/ml) were then mixed with the appropriate inhibitor and immediately plated out on to the relevant FITC-labelled collagen substrate. 50µl of supernatant were

then removed at specific time points and analysed, as described in section 2.2.5.5 using an excitation and emission wavelength of 492nm and 535nm, respectively, in a SpectraFluor® plate reader

2.2.1.11. Inhibition of MMP activity: Effect in cell-matrix remodelling

Stock solutions of the MMP broad-range inhibitor GM6001 (Galardin; 364205) and the corresponding negative control (364210) (CalBiochem, Nottingham, UK) were prepared according to the manufacturers' instructions. 5mg/ml of FITC-labelled collagen was incubated overnight at 37°C, alone and with, either treatment with 50µg/ml of tTG or 50µg/ml mTG. 2ml of cell-suspension (2 x 10^5 cells/ml), mixed with the appropriate inhibitor (final concentration; 25µM GM6001 and 25µM of the commercial GM6001 negative control), was plated out on to the relevant FITC-collagen substrates and maintained in a humidified-atmosphere incubator at 37°C and with 5% (v/v) CO_2. 75µl of the cell culture supernatant was removed at specific time points and analysed, as described in section 2.2.5.5, using an excitation and emission wavelength of 492nm and 535nm, respectively, in a SpectraFluor® plate reader.

2.2.2. TRANSGLUTAMINASE

2.2.2.1. Tissue transglutaminase

2.2.2.1.1. Isolation and purification

Tissue transglutaminase (mammalian transglutaminase; tTG) was isolated and purified from guinea pig livers using a combination of anion exchange, gel filtration and affinity chromatography as previously described by [LeBlanc *et al.,* 1999] with several modifications as follows:

2.2.2.1.2. Liver homogenisation

At least six guinea pigs (Winchester strain; ex-breeders) were freshly sacrificed by neck dislocation and their livers (200-250g) extracted and homogenised to a 50% (w/v) homogenate in two steps; firstly on a bench-top blender in 500ml of ice-cold buffer consisting of 5mM Tris-HCl, 2mM EDTA, pH 7.5 and 0.25M sucrose, supplemented with the following protease inhibitors: 5mM benzamidine, 1mM PMSF and 10µg/ml leupeptin hydrochloride, before then being subjected to

multiple cycles on a bench-top homogeniser. Cell nuclei and large debris were spun out by centrifugation at 30000g for 1 hour, at 4°C, in a Beckman centrifuge (Beckman, High Wycombe, UK).

2.2.2.1.3. Anion-exchange chromatography

The supernatant was collected and cellular debris and membranes removed by a further clarification step at 100000g for 1 hour, at 4°C, using multiple runs in an ultracentrifuge. This resulting supernatant was further filtered, at 4°C, through a series of filters consisting of: a dual layer of 10 dernier ladies tights, Whatman no.42 filter paper (Whatman, Maidstone, UK) and a final step with Whatman no.1 filter paper before being loaded onto a 300ml Q-Sepharose Fast Flow column (Amersham Pharmacia, Buckinghamshire, UK) using a two stage gradient set-up of: 0-36% 'Buffer B' for 95 minutes followed by 36-72% 'Buffer B' for a further 95 minutes. This whole procedure was performed in a chilled-cabinet, at 4°C. The mobile phase for this step comprised of 'Buffer A' consisting of 5mM Tris-HCl, 2mM EDTA, pH 7.5 and 'Buffer B' consisting of 5mM Tris-HCl, 2mM EDTA, pH 7.5, 1M NaCl. Following loading of the sample, the column was washed through with 'Buffer A' until the UV absorbance dropped to 0.5units (approximately 2 column volumes) with a set-up run at 15ml/min via an Econosystem pump. Proteins were eluted with a linear gradient of 0-720mM NaCl in 'Buffer A' over 192 minutes, with collection of 15ml per fraction. The TG activity and protein profile were determined by the hydroxamate activity assay and Lowry protein assay, respectively.

2.2.2.1.4. Gel filtration chromatography

The high activity TG rich fractions, from the anion-exchange chromatography procedure, were pooled together and precipitated by the gradual addition of ammonium sulphate to 80% (w/v), on ice for 45 minutes with constant buffering using 1M Tris-HCl, pH 7, to a neutral pH 7. This precipitate was then split equally into four aliquots before being spun down in a MSE 24 centrifuge (MSE, Cambridge, UK) at 30000g, for 1 hour, at 4°C. Pellets resulting from each aliquot (equivalent to 25% of the total protein) were reconstituted in 'running buffer' consisting of 10ml of 50mM Tris-acetate, 1mM EDTA, 0.16M KCl, 1mM DTT, pH 6.0, before being loaded onto a 2.5x100cm Sephracryl S-200 gel filtration column (Bio-Gel A-0.5m, fine mesh) at a flow rate of 2ml/min in the same buffer.

The elution was conducted, at 4°C, for a total of 336 minutes with the collection of 5ml fractions between 67 and 246 minutes. The TG activity and protein profile were determined by the hydroxamate activity assay and Lowry protein assay, respectively.

2.2.2.1.5. GTP-agarose affinity chromatography

The high specific TG activity fractions were applied to a 5ml bed volume GTP agarose column (Sigma, Poole, UK) equilibrated in 50mM Tris-acetate, 1mM EDTA, 1mM DTT, pH 7.5 and run through at 2ml/min using a peristaltic pump, at room temperature. The flow-through was then reloaded to ensure saturation of the binding sites. The column was then washed with 100ml of the same buffer before elution with 5mM GTP in 50mM Tris-HCl, 1mM EDTA, and 1mM DTT, pH 7.5 with 1ml fractions being collected. The resulting fractions were assayed for TG activity and protein content by the biotin-cadaverine and Lowry protein assays, respectively. Furthermore, purity was also verified by SDS-PAGE and western blotting with an anti-tTG MAb and compared to that of the commercially available tTG from guinea pig liver (Sigma, Poole, UK).

2.2.2.1.6. Dialysis and storage

Following gel filtration, GTP contamination was removed by overnight dialysis with three buffer changes at 4°C of PBS/2mM EDTA, pH 7.4 using dialysis tubing with a 5kDa MW cut-off point (Perbio Science, Cheshire, UK). Samples were then snap-frozen in liquid nitrogen and lyophilised before being stored at -70°C for long-term storage.

2.2.2.2. Microbial transglutaminase

2.2.2.2.1. Isolation and purification

The microbial transglutaminase (mTG; isolated from *Streptoverticillium mobaraense*) used throughout this investigation was from the commercially available product, Activa™ WM (Ajinomoto Corporation Inc. Japan) as a 1% preparation combined with maltodextrin. A single step purification step was required to remove this extraneous maltodextrin ingredient before use. Approximately 50g of the Activa™ WM was dissolved in ice-cold buffer

consisting of 20mM sodium phosphate and 2mM EDTA, pH 6.0, before being filtered through Whatman no.1 filter paper, and then loaded onto a 100ml SP-Sepharose FF column overnight at a continuous flow rate of 5ml/min. The column was then washed with a minimum of 5 column volumes of the same buffer before protein elution was performed, under the same flow rate, with a linear 0-1000mM gradient of ice-cold buffer consisting of NaCl, 20mM phosphate buffer and 2mM EDTA, pH 6.0. The resultant collected fractions were then assayed for TG activity and protein content by the hydroxamate and Lowry protein assays, respectively. Furthermore, purity was also verified by SDS-PAGE and western blotting with an anti-mTG MAb (N-Zyme Biotech, Darmstadt, Germany). Fractions containing high-activity mTG were pooled and lyophilised before being stored at -70°C for long-term storage.

2.2.2.3. Transglutaminase activation and inhibition

Tissue transglutaminase was activated by treatment with 5mM DTT and 5mM $CaCl_2$, in 10mM Tris-HCl, pH 7.4 buffer before immediate use. 10mM EDTA was used as the standard reaction inhibitor. The synthetic CBZ-glutaminyl-glycine analogues, R281 and R283, (kindly synthesised by Dr Saints and Dr. Coutts, School of Chemistry, Nottingham Trent University, UK) were also used as irreversible site-directed TG-inhibitors [Balklava, 2002; Freund *et al.*, 1994] at concentrations of, at least 500mM with an incubation time of 30 minutes at 37°C.

2.2.2.4. Transglutaminase activity assay

Three different techniques were used to determine the activity of the transglutaminases in the different applications.

2.2.2.4.1. Incorporation of [^{14}C]-putrescine into N,N'-dimethylcasein

Originally described by Lorand and co-workers, this assay relies on the enzymes ability to catalyse the incorporation of a radioactively labelled primary amine into a protein acceptor substrate [Lorand *et al.,* 1972]. Briefly, at 30 second intervals, 45µl of sample was added to 45µl reaction mix containing 10µl 50mM Tris-HCl, pH 7.4, 10µl 38.5mM DTT, 10µl 12mM [1,4-^{14}C]-putrescine, 20µl 25mg/ml N,N'-dimethylcasein in 50mM Tris-HCl and either 5µl 50mM $CaCl_2$ or 5µl 200mM

EDTA pre-equilibrated at 37°C in a water-bath. After 15 minutes of the initial commencement of the reaction, triplicate 10µl aliquots from each sample were removed and spotted onto $10mm^2$ of Whatman 3MM filter paper at 30 second intervals. The squares were then placed in 10% (w/v) ice cold TCA for 10 minutes, washed consecutively three times for 5 minutes in 5% (v/v) TCA, once in acetone: ethanol (1:1, v/v) for 5 minutes and once in acetone for another 5 minutes. The filter papers were then air dried for 15 minutes and, once dry, were placed in scintillation tubes, to which 2ml Ultima Gold™ scintillation fluid (Packard Biosciences, Pangbourne, UK) was added and the radioactivity counted with a Packard Liquid Tri-Carb LS counter (Packard Biosciences, Pangbourne, UK). A unit of transglutaminase activity is defined as 1nmol of putrescine incorporated per hour.

2.2.2.4.2. Biotin-cadaverine incorporation into N,N'-dimethylcasein

This TG-activity assay is based on a modification of the technique originally described by Slaughter and co-workers [Slaughter *et al.,* 1992]. Briefly, 96-well plates were coated with 250µl of 100mg/ml N,N'-dimethylcasein in 100mM Tris-HCl, pH 8.5 for 16 hours at 4°C.The plates were then washed twice with PBS, 0.05 % (v/v) Tween20 and twice in distilled H_2O before being blocked with 250µl of 3% (w/v) BSA in 100mM Tris-HCl, pH 8.5 for 30 minutes at room temperature. Plates were once again washed 3 times with PBS, 0.05 % (v/v) Tween20 and once more with 100mM Tris-HCl pH 8.5. The reaction was initiated by addition of 50µl of transglutaminase-containing solution and 150µl of 0.1mg/ml biotin-cadaverine in 100mM Tris-HCl pH 8.5, 13.3mM DTT, supplemented with 6.67mM $CaCl_2$, or with 10mM EDTA- as the positive and negative control samples respectively. The plates were then incubated at 37°C for 30 minutes, and the reaction was terminated with 10mM EDTA and then washed as described above. Biotin-cadaverine incorporation into N,N'-dimethyl was detected by incubation for 1 hour at 37°C with 200µl of an extravidin peroxidase conjugate diluted 1:5000 in blocking buffer (3% (w/v) BSA in 100mM Tris-HCl, pH 8.5). Colour development was achieved in a phosphate-citrate buffer with urea-H_2O_2 (one phosphate-citrate tablet with urea-H_2O_2 in 10ml distilled water; Sigma) containing 7.5% 3,3',5,5'-tetramethylbenzidine (TMB). The reaction was terminated by the addition of 2.5N H_2SO_4 and the resultant absorbance was read at 450nm in a SpectraFluor® plate reader.

2.2.2.4.3. Hydroxamate assay

The hydroxamate assay used involved a slight modification of the method developed by Folk and Chung [Folk and Chung, 1985].

Reagent A	Reagent B
0.2M sodium acetate (pH 6.0)	1 volume 3N HCl
0.03M CBZ-Gln-Gly	1 volume 12% trichloroacetic acid
0.1M hydroxylamine	1 volume 5% $FeCl_3.6H_2O$ (dissolved in 0.1 N HCl)
0.01M glutathione	
* 0.005M $CaCl_2$	
* 0.005M DTT	

* only applicable in determination of tTG activity

In short, a 25µl portion of sample was mixed with 75 µl of Reagent A in a well of a 96-well plate. Following incubation at 37 °C for 10 minutes, 75 µl of Reagent B was added to the well in order to terminate the reaction and by the formation of an iron complex. The resultant absorbance was read at 492nm in a SpectraFluor® plate reader. A unit of transglutaminase activity is defined as the amount of enzyme catalyzing the formation of 1µmol of hydroxamic acid per minute under the described reaction conditions.

2.2.3. COLLAGEN

2.2.3.1. Isolation and purification

Collagen type I was sourced from calf tendons and rat tails as follows: tendons were dissected and removed from freshly obtained rat tails or calf tendons and extracted with 0.017M acetic acid with constant stirring at 4°C for 48 hours. The solution was then centrifuged at 13000g for 1h at 4°C to remove insoluble non-collagenous material, before being adjusted to pH 7 with 1M NaOH and stirred for a further 2 hours, at 4°C, to allow the precipitation of the collagen. This collagenous-suspension was then centrifuged at 8000g for 20 minutes at 4°C in a Beckman centrifuge (Beckman, High Wycombe, UK) with the resulting collagen pellets being stored at -20°C until required. A commercial source of collagen type III (calf skin) was also used in the investigation.

2.2.3.2. Collagen neutralisation

Native collagen samples were solubilised in 0.2M acetic acid at 4°C with constant stirring for 24 hours before use. Neutralisation of the collagen mixture was performed using a [7:1:1:1] ratio of [collagen: 10X DMEM: 10X PBS: 1M NaOH] respectively to a final of pH 7.2. Tissue culture plastic was then covered using this collagen mix (recommended at 6-10µg/cm^2) before being placed into a humidified-atmosphere incubator overnight to allow gelation to occur. In general, 50µl of the collagen mix was added to each well of a 96 well plate. Plates were used within 48 hours of the collagen matrix formation.

2.2.3.3. Modification of collagen by transglutaminase

Neutralised collagen mixture was subjected to treatment with both tTG and mTG: 50-1000µg/ml of tTG, in a reaction mix consisting of 5mM DTT and 5mM $CaCl_2$ in 10mM Tris buffer (pH 7.4) or mTG in 10mM Tris buffer (pH 7.4) were directly added the collagen upon neutralisation as described in Section 2.2.3.2. Stock solutions of: 2mg/ml tTG and mTG, 1M DTT and 1M $CaCl_2$ were used to minimise total volume changes. The enzymes were always added last to the collagen-reaction mix to minimise any self-imposed cross-linking. For 96 well plates, 50µl of the pre-treated collagen mixture was added to each well before being placed into a humidified-atmosphere incubator, at 37°C and with 5% CO_2, overnight. On removal, wells were washed twice with sterile distilled water and used immediately.

2.2.3.4. Incorporation of peptides and fibronectin into collagen matrices

Novel synthetic peptides and fibronectin were further incorporated into neutralised collagen via the use of TG. Stock concentration (1mg/ml) of the synthetic peptides; EP011803 (H2N-EAQQIVPHSRNGGGRGD-COOH) and EP011804 (H2N-GKKGKKGPHSRNGGGRGD-COOH) (Eurogentec, Southampton, UK) and the FITC-labelled peptides FITC-TVQQEL and FITC-KKKKGY (Alta Bioscience, University of Birmingham, UK) were prepared according to the manufacturers' instructions. A 1mg/ml stock concentration of human plasma fibronectin (Sigma, Poole, UK) was reconstituted in distilled water according to the manufacturers' instructions. Immediately following the neutralisation of collagen, as described in the section 2.2.3, the corresponding amount of peptide or fibronectin (FN) was added to the mixture, followed by 5mM

DTT, 5mM $CaCl_2$ in 10mM Tris buffer (pH 7.4) and then 200µg/ml of tTG. In the case of treatment with mTG, 200µg/ml of the enzyme was added by itself i.e. without DTT and $CaCl_2$. Samples were then placed, overnight, into a humidified-atmosphere incubator, at 37°C and with 5% CO_2. On removal, wells were washed twice with sterile distilled water and used immediately.

2.2.3.5. Detection of incoporated FITC-labelled peptides

Stock concentration (1mg/ml) of the FITC-labelled peptides FITC-TVQQEL and FITC-KKKKGY (Alta Bioscience, University of Birmingham, UK) were prepared according to the manufacturers' instructions and incorporated into neutralised collagen via the use of differing concentrations of TG, as described in section 4.2.1. Samples were then placed, overnight, into a humidified-atmosphere incubator, at 37°C and with 5% CO_2. On removal, the samples were washed twice with sterile distilled water and then treated with 100µl of 1mg/ml microbial collagenase solution (*Clostridium histolyticum*), followed by 100µl 0.25% (w/v) trypsin/2mM EDTA solution in PBS, pH 7.4, for 24 hours at 37°C, with constant shaking. 250µl aliquots were removed at selective time-points to a 96 well-plate and the fluorescence measure using an excitation and emission wavelength of 492nm and 535nm, respectively, in a SpectraFluor® plate reader.

2.2.4. MECHANICAL AND PHYSICAL CHARACTERISATION

2.2.4.1. Atomic force microscopy (AFM)

2.2.4.1.1. Slide preparation

Neutralised and treated samples were added directly on to untreated -uncoated glass slides (BDH, Poole, UK), incubated/dried at 37°C for 2 hours before being washed, once, with distilled water and then left on the bench to air-dry for a further 30 minutes.

2.2.4.1.2. AFM testing

A Dimension 3100 atomic force microscope (Veeco Instruments Ltd., Cambridge, UK), utilising 'tapping mode' settings with a drive frequency close to the resonance frequency of the cantilevers, was used in the imaging of the

samples. Imaging was conducted simultaneously in the height mode and phase mode and, in order to obtain an appropriate force between the sample and the tip (to ensure the tip was tracking the surface properly), the ratio of the set point value to the free amplitude of the cantilever was maintained at approximately 1.0. Scan size was set at 0.8-8μm (scan rate, 1Hz) or 6-20μm (scan rate, 0.3Hz) for collagen-only or collagen-cell samples, respectively. The drive amplitude of the cantilever was set to a root mean square (RMS) value of 18-30nm. All samples were viewed and imaged at room temperature. Captured images were achieved using the in-built digital camera and further analysed with the Nanoscope software platform.

2.2.4.2. Rheological studies

The elastic (G') and viscous (G") moduli were determined with a Bohlin C-VOR KTB30 rheometer (Malvern Instruments, Worcestershire, UK), fitted with a cylindrical Couette geometry set-up and 2cm diameter serrated plates. The, *in situ,* fibrillogenesis characteristics were also monitored but with the use of 2cm diameter smooth plates. In all cases, the plate-gap dislocation distance was set at 0.8mm and sample conditions maintained at 37°C using a circulating water bath set-up.

2.2.4.3. Free-radical scavenging (antioxidant activity)

A slightly modified version of the direct decolourisation assay described by Re and co-workers was used to measure the total antioxidant activity of samples based on their free-radical scavenging ability [Re *et al.*, 1999]. Briefly, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was dissolved in distilled water to yield a 7mM solution. The initiation of the ABTS radical cations ($ABTS^{\bullet+}$) was produced by reacting this stock solution with 2.45mM potassium persulphate (final concentration) and incubating, at room temperature, in the dark for 16 hours before use. The $ABTS^{\bullet+}$) solution was further diluted with PBS, pH 7.4, to an absorbance of approximately 0.700 at 734nm, at room temperature, using a DU-7 spectrophotometer (Beckman Instrument (UK) Ltd, High Wycombe, UK). Each of the samples were then dissolved in PBS, pH 7.4, and then 10μl of each was thoroughly mixed with 1.0ml of diluted $ABTS^{\bullet+}$ solution. This reaction was allowed to proceed for exactly 1-3 minutes, at room temperature, with constant shaking, after which the

samples were centrifuged at 13000g for 30 seconds and the supernatants retained. Free-radical scavenging activity of the mixed solutions was then determined by decolourisation of $ABTS^{\bullet+}$, through measuring the reduction of the radical cation as percentage inhibition of absorbance at 734nm.

2.2.4.4. Fibrillogenesis (turbidity assay)

Collagen fibrillogenesis was monitored using a spectrophotometric method as described by Nomura and co-workers [Nomura *et al.,* 2000]. In short, the neutralization of (native and modified) collagen samples, as previously described, was performed, *in situ,* using silica cuvettes at 325nm wavelength (0.3mm slit width, 1.0nm bandwidth) in a PYE Unicam Recording SP1800 UV spectrophotometer. Temperature was maintained at 25°C using a Techne C-85A circulator water bath set-up.

2.2.5. BIOCHEMICAL ANALYSIS, DETERMINATION AND IDENTIFICATION

2.2.5.1. Determination of ε-(γ-glutamyl)lysine cross-link

The amount of the ε(γ-glutamyl) lysine cross-link dipeptide was quantified as outlined previously by Griffin and Wilson [1984] involving intensive proteolytic digestion and analyses by ion-exchange chromatography. Briefly, cross-linked and native samples of collagen were homogenised to yield a 20% (w/v) homogenate in 0.2M sucrose, 2mM EDTA, 5mM Tris-HCl, pH 7.4. Following the addition of 100% (v/v) trichloroacetic acid to a final concentration of 10% (v/v), samples were then centrifuged at 4,000g at 4°C, for 15 minutes. Protein rich pellets were then washed once in diethyl ether/ethanol (1:1) and another three times with diethyl-ether only and air-dried at room temperature before being resuspended in 50μl of 0.1M $(NH_4)HCO_3$ with the addition of (a single crystal of) thymol. After resuspension of the pellets by sonication, several sequential digestion of proteins were performed using a series of proteolytic enzymes (10μg/ml subtlisin, 3x18 hours, at 37°C; 10μg/ml microbial collagenase, 18 hours, at 37°C; 15μg/ml pronase, 12 hours, at 32°C, 10U leucine aminopeptidase/15U prolidase, 2x10 hours, at 37°C; and 20μg/ml carboxypeptidase, 12 hours, at 30°C; all Sigma-Aldrich) to release the ε(γ-glutamyl) lysine crosslink dipeptide. After digestion, samples were freeze dried and then resuspended in 0.1M HCl and sonicated for 2min to aid dispersion. An

aliquot (90µl) was mixed with 110µl of loading buffer (0.2M lithium citrate, 0.1% phenol pH 2.2) and loaded onto a Dionex DC-4A resin column 0.5cm x 20cm using a Pharmacia Alpha Plus amino acid analyser.

Time (min)	Buffer*	Column Temperature
0-9	1	25°C
9-32	2	25°C
32-67	3	25°C
67-107	3	25°C
107-123	6	75°C
123-135	1	75°C
135-147	1	65°C
147-159	1	35°C
159-171	1	25°C

* Buffer 1: 0.2M lithium citrate, 0.1% phenol, 1.5% (v/v) propan-2-ol pH 2.8.
Buffer 2: 0.3M lithium citrate, 0.1% phenol, 1.5% (v/v) propan-2-ol pH 3.0.
Buffer 3: 0.6M lithium citrate, 0.1% phenol pH 3.0.
Buffer 6: 0.3M lithium hydroxide.

Derivatisation was performed post column using o-pthaldialdehyde (0.8M boric acid, 0.78M potassium hydroxide, 600mg/ml o-phthaldialdehyde, 0.5% (v/v) methanol, 0.75% (v/v) 2-mercaptoethanol, 0.35% (v/v) Brij 30) and the absorbance was measured at 450nm. The dipeptide was determined by addition of known amounts of ε(γ-glutamyl)lysine standard to the sample and comparing peak areas.

2.2.5.2. Quantification of amino acid content by gas chromatography

Samples were homogenised and sonicated in a 0.2M sucrose, 2mM EDTA, 5mM Tris-HCl, pH 7.4, buffer and supplemented with protease inhibitors (1µg/ml pepstatin, 1µg/ml leupeptin, 1mM phenylmethylsulphonyl fluoride) to a final concentration of 200mg/ml (w/v). Hydrolysis of 50µl of the sample homogenates was performed in 6M HCl at 120°C, for 16 hours, prior to further evaporation of residual liquid by incubation at 50°C for 48 hours. The dried pellets were then resuspended in 500µl of 50mM Tris, pH 8.0. Amino acids were separated following a solid-phase extraction/derivatisation protocol using the EZ-Faast™ amino acid analysis kit (Phenomenex, Macclesfield, UK) according to the manufacturer's instructions. The amino acid and hydroxyproline content of the samples were measured against internal amino acid standards (5-20nmoles) by gas chromatography using a 9890N Network GC system (Agilent Technologies UK Ltd., Stockport, UK) running under constant flow mode; 1:15 split injection at

250°C, 2.5µl; using helium as the carrier gas at 60kPa, with 3kPa/min increments. The oven was programmed for 32°C/min from 110°C to 320°C, holding at 320°C for 1 minute, before detection at 320°C. The hydroxyproline content was expressed as percentage of total amino acids present.

2.2.5.3. Coomassie blue staining of matrices

The capacity of both the HOB and HFDF cells to degrade type I collagen was assessed as previously described by Holmbeck *et al.*, [1999]. Briefly, native and TG pre-treated collagen samples gels were plated out at 50µl per well of a 96 well plate. 100µl of 2 $x10^4$ cells/ml, in culture medium, was then added to the wells in triplicates. Plates were then kept at 37°C and 5% (v/v) CO_2, in a humidified-atmosphere incubator, for the relevant time point(s). After incubation, the cells were removed from the collagen matrix by addition of 0.5% (w/v) sodium-deoxycholate in 10mM Tris-HCl and constant visual monitoring. A final rinse with distilled water was performed before the collagen samples were stained with a 0.1% (w/v) Coomassie Brilliant blue stain solution (50% (v/v) methanol; 10% (v/v) acetic acid; 40% (v/v) dH_2O). The samples were allowed to stain for 5 minutes before another rinse with distilled water. Unstained areas, which appeared lighter blue, gave an indication of collagen degradation by cells. Two separate fixed-size random fields per triplicate samples were photographed using an Olympus CK2 microscope and pictures taken with a DP10 digital camera.

2.2.5.4. Degradation of matrices by microbial collagenase

The collagen substrates were subjected to a simple proteolytic digestion treatment. Samples were washed twice with PBS, pH 7.4, followed by another wash with distilled water before commencement of the enzymatic treatment which consisted of the addition of 100µl of a 1mg/ml microbial collagenase solution (*Clostridium histolyticum)* followed by treatment with 100µl 0.25% (w/v) trypsin/2mM EDTA solution, in PBS pH 7.4, solution for 24 hours at 37°C. The residual total protein concentration was determined as described in Section 2.2.4.

2.2.5.5. Degradation of FITC-labelled matrices by purified MMPs and cell culture supernatant

5mg/ml of FITC-labelled collagen (Merck Biosciences, Hertfordshire, UK) was incubated overnight at 37°C with shaking in 50mM Tris-HCl pH 7.4, 5mM $CaCl_2$, 5mM DTT, alone and with, either treatment with 0.5mg/ml of tTG or 0.5mg/ml mTG. The collagen was pelleted by centrifugation (13000g, 5 minutes) and washed twice with 1ml of 50mM Tris-HCl pH 7.4, 5mM $CaCl_2$, before being resuspended in 1ml of 50mM Tris-HCl pH 7.4, 5mM $CaCl_2$ containing: 50ng/ml of purified MMP1 or MMP8 in 50mM Tris HCl pH 7.4, 5mM $CaCl_2$, 0.005% Brij-35, 1µM $ZnCl_2$; 20µg/ml *Clostridiopeptidase A* or supernatant from HFDF cells (48h culture period) used at a dilution of 1/20 in 50mM Tris-HCl pH 7.4, 5mM $CaCl_2$. Digestion was performed at 37°C with constant shaking, from which 100µl aliquots were continuously removed to a 96-well plate, at half-hourly time points, and the fluorescence measured using an excitation and emission wavelength of 492nm and 535nm, respectively, in a SpectraFluor® plate reader.

2.2.5.6. Integrin mediated cell adhesion inhibition

The function blocking of integrins was achieved using antibodies provided in the Collagen-Specific Integrins Investigators kit (Chemicon, Hampshire, UK). Antibodies were prepared and used according to the manufacturers' instructions with the optimised dilution factors determined experimentally. Cell samples (2 x 10^5 cells/ml) were then mixed with the appropriate antibody before being immediately plated out on to the relevant substrate. Samples were then cultured in humidified-atmosphere incubator, at 37°C and with 5% CO_2, for the desired time points, before being subjected to the spreading and attachment analysis as described in section 2.2.1.5.

2.2.5.7. RGD-mediated cell adhesion inhibition

Stock solutions of the synthetic peptides GRGDTP and GRADSP (CalBiochem, Nottingham, UK) were prepared in DMEM according to the manufacturers' instructions. Cell samples (2 x 10^5 cells/ml) were then incubated in a humidified-atmosphere incubator, at 37°C and with 5% CO_2, with the corresponding peptide for 15 minutes, before being plated out on to the relevant substrate and subjected to the spreading and attachment analysis as described in section 2.2.1.5.

2.2.5.8. MMP and TIMP expression by microarray

The multiple detection of MMPs and TIMPs was achieved using the commercially available RayBio® Human Matrix Metalloproteinase Antibody Array I kit (Insight Biotechnology Ltd., Middlesex, UK) with the samples treated according to the manufacturers' instructions. Samples were normalised for protein concentration prior analysis. Briefly, membranes were blocked with a 1X Blocking Buffer for 30 minutes, at room temperature, before its removal and the subsequent addition and incubation of 1ml of corresponding samples for 2 hours. The samples were then removed and the membranes washed 3 times, for 5 minutes each time, with 2ml of Wash Buffer I, at room temperature, with constant shaking. Another two washes were then performed with Wash Buffer II, for 5 minutes each time, at room temperature and with constant shaking, after which, 1ml of the diluted biotin-conjugated antibody was added to each membrane. These were then incubated for 2 hours at room temperature. Samples were then washed as previously described with Wash Buffers I and II before 2ml of the diluted HRP-conjugated streptavidin solution was added to each membrane and incubated for a further 2 hours at room temperature. Detection was achieved by combining the provided detection reagents, pipetting on to each membrane and incubating for 2 minutes at room temperature. Excess detection reagent was blotted against lint-free tissue, before the membranes were covered using the provided plastic sheeting and fixed, with tape, in a Kodak Biomax exposure cassette. The resulting light emission was detected by exposure to Kodak X-Omat chemiluminescence detection film (Roche Diagnostics, East Sussex, UK) for 1-5 minutes, depending on the intensity of the signal. The film was developed using 20% (v/v) LX-24 developer, fixed in 20% (v/v) FX-40 fixer and finally rinsed with distilled water before being air dried on the laboratory bench.

2.2.6. PROTEIN ANALYSIS, DETERMINATION AND IDENTIFICATION

2.2.6.1. Protein concentration

The total protein content of samples was determined using either the Lowry protein assay [Lowry *et al.*, 1951] or the Bicinchoninic acid (BCA) assay [Brown *et al.*, 1989]. The choice of assay was dependent on their compatibility with reducing agents, detergents, or other supplements contained in the solubilisation or sample buffers.

2.2.6.1.1. Lowry protein assay

The Lowry protein assay was performed using the Bio-Rad RC kit (Life Sciences, Hemel Hempstead, UK) according to the manufacturers' instructions and summarised as, simply, the sequential addition of 25µl of reagent A and 200µl of reagent B, to 5µl of sample or, to BSA standards ranging from 0.15-10mg/ml in a 96-well plate format. The plate was incubated for 10 minutes at room temperature before the absorbance was read at 750nm using a SpectraFluor® plate reader.

2.2.6.1.2. BCA protein assay

The BCA assay was used when protein samples from the investigation contained substrates that were incompatible with the standard Lowry assay. Proteins were precipitated and delipidated, at the same time, by addition of a mixture of ice cold acetone, tri-n-butyl-phosphate in a 1:1:12 ratio, followed by storage at -70°C for 45 minutes, and subsequent centrifugation at 13,000g for 10 minutes, as described by Mastro and co-workers [Mastro *et al.*, 1999]. Pellets were then washed in tri-n-butyl-phosphate, acetone, and methanol before being reconstituted in 1% (w/v) SDS. The BCA reagent, prepared freshly was obtained by mixing solution A [1% (w/v) bicinchoninic acid in sodium salt form, 2% (w/v) sodium carbonate, 0.16% (w/v) sodium tartate, 0.4% (w/v) sodium hydroxide, 0.95% (w/v) sodium hydrogen carbonate, pH 11.5) with solution B [4% (w/v) copper sulphate] at a 25:1 ratio. A 5µl sample of the protein suspension was then mixed with 200µl of this BCA reagent. Protein concentrations were determined against a parabolic standard curve produced by triplicate BSA protein standards (0.15-10mg/ml) using the same methodology. The plate was incubated for 30 minutes, at 37°C, before the absorbance was read at 562nm using a SpectraFluor® plate reader.

2.2.6.2 Preparation of total cell lysates

Cell-lysate samples, for the analysis of membrane-bound proteins, were obtained by the following techniques. The choice of assay was dependent on its compatibility with the specific assay.

2.2.6.2.1. Laemmli solubilisation

The cell culture medium was removed from samples cultured on the relevant substrate and the cells washed, *in situ*, once with room-temperature-PBS pH 7.4 and then, once more, with ice-cold PBS pH 7.4. A 6x Laemmli solubilisation buffer (375mM Tris-HCl pH 6.8, 9% (w/v) SDS, 50% (v/v) glycerol, 9% (v/v) 2-β-mercaptoethanol, 0.03% (w/v) bromophenol blue) was added directly on to the sample and then incubated for 15 minutes at room temperature. Following incubation, the samples were sheared by multiple passes through a 20-gauge and incubated for a further 30 minutes at room temperature and then centrifuged at 10000g for 20 minutes. The resulting supernatant (total cell lysate) was then transferred to a new tube and stored, at -20°C, until required.

2.2.6.2.2. Lysis buffer

The cell culture medium was removed from samples cultured on the relevant substrate and the cells washed, *in situ*, once with room-temperature-PBS pH 7.4 and then, once more, with ice-cold PBS pH 7.4. The following steps were performed on ice: 0.6ml of RIPA lysis buffer (Santa Cruz, Calne, UK) per 100mm culture area was added directly to the samples and gently rocked for 15 minutes at 4°C. Following incubation, the samples were sheared by multiple passes through a 20-gauge needle and incubated for a further 1 hour, on ice, and then centrifuged at 10000g for 15 minutes at 4°C. The resulting supernatant (total cell lysate) was then transferred to a new tube and stored, at -20°C, until required.

2.2.6.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The method used was based on a modification of that described by Laemmli for use with standard vertical slab gel apparatus (Laemmli, 1970). Gels were cast using the Atto-minigel system (B & L Systems, Marseen, The Netherlands) and consisted of a standard 3% (w/v) polyacrylamide stacking gel and a 5-15% (w/v) resolving gel- dependent on the application required. The acrylamide stock solution used for all gels consisted of 30% (w/v) acrylamide and 0.8% (w/v) N, N′-methlylene bisacrylamide. Stacking gels were made using a Tris-SDS stock solution, pH 6.8 [0.25M Tris, 0.2% (w/v) SDS) whereas resolving gels contained

a Tris-SDS solution pH 8.8 [0.75M Tris, 0.2% (w/v) SDS]. The recipes for different concentrations of acrylamide in the resolving gel are listed in Table 2.2.4.2. Resolving gels (80 x 60x 0.75mm) were cast using the Atto-system according to the manufacturer's protocol.

Stock solutions	Final acrylamide concentration in the separating gel (%) (ml)									
	5	6	7	7.5	8	9	10	12	13	15
30% acrylamide/ 0.8% bisacrylamide	2.50	3.00	3.50	3.75	4.00	4.50	5.00	6.00	6.50	7.50
4x Tris-HCl/SDS (pH 8.8)	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
H_2O	8.75	8.25	7.75	7.50	7.25	6.75	6.25	5.25	4.75	3.75
10% ammonium persulphate	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TEMED	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

A layer of iso-propan-2-ol was poured on the top of the gel to an approximate depth of 5mm to provide the gel with a flat upper surface, following which the gel was allowed to polymerise for, at least, 40 minutes at room temperature. The upper surface of polymerised resolving gels was washed twice with distilled water to remove any residual iso-propan-2-ol. Finally, the edge of the gel was gently blotted dry using Whatman no. 1 filter paper. Stacking gels were prepared by combining 0.65ml of the 30% (w/v) acrylamide and 0.8% (w/v) N, N'-methlylene bisacrylamide solution, 1.25ml of Tris/SDS pH 6.8 and 3.05 ml of distilled water. Polymerisation was initiated by the addition of 25µl 10% (w/v) ammonium persulphate and 5µl of TEMED, before this mixture was quickly pipetted between the glass plates with the appropriate comb inserted to form the sample wells. Polymerisation was allowed to proceed for a minimum of 45 minutes at room temperature, after which the sample well comb was gently removed and the wells washed and filled with Tris-glycine electrode running buffer, pH 8.5 (0.025M Tris, 0.192M glycine and 0.1% (w/v) SDS). Sample volumes to be loaded were normalised against protein concentration (approximately 15-20µg) combined with the appropriate volume of 2x Laemmli loading buffer (125mM Tris-HCl, 20% (v/v) glycerol, 4% (w/v) SDS, 2% (v/v) mercaptoethanol and 0.02 mg/ml bromophenol blue) before being loaded into the sample wells using specialised protein

electrophoresis loading tips (Bio-Rad, Hertfordshire, UK). Electrophoresis was performed at 150V for approximately 1.5 hours and terminated when the bromophenol blue marker dye front was about to escape from the bottom of the gel.

2.2.6.3.1. Coomassie blue staining

Gels were removed from the apparatus and stained using a Coomassie blue staining solution (0.2% (w/v) Coomassie blue R-250, 45% (v/v) methanol, 10% (v/v) acetic acid) and destained with destain solution (10% (v/v) methanol, 10% (v/v) acetic acid).

2.2.6.4. Western blotting of SDS-PAGE separated proteins

The electrophoretic transfer of SDS-PAGE separated proteins to a nitrocellulose membrane was performed as described by Towbin and co-workers [Towbin *et al.,* 1979] using a Protean II Cell Systems wet-blot system (Biorad, Hertfordshire, UK). Residual SDS was removed from the gel by continuous washing in transfer buffer (48.8mM Tris-HCl, 39mM glycine and 20% (v/v) methanol) for 10 minutes on a shaker-platform. The nitrocellulose transfer membrane (Gelman Biosciences, Northampton, UK), scanting pads and blot-specific absorbent filter paper (Biorad, Hertfordshire, UK) were also equilibrated in the same transfer buffer. The whole apparatus was assembled according to the manufacturers' instructions; with the scanting pad on the bottom, followed by the filter paper, the sample gel, the nitrocellulose membrane, another piece of filter paper and then, finally, with another scanting pad. During the assembly procedure, any air bubbles trapped in between the filter papers and the membrane were removed by the rolling action with a glass rod. Western blotting was carried out using the Biorad apparatus and power supply for, a minimum of, 90 minutes at 150mA in pre-chilled transfer buffer. Once transfer was completed, the equipment was dissembled and the nitrocellulose membrane was separated from the gel and placed in a plastic tray. Transfer of proteins was verified by staining with Ponceau Red solution (0.2% (w/v) Ponceau S, 0.4% (v/v) glacial acetic acid) and subsequent destaining in distilled water. Residual stain was further removed during the following blocking step. Washed blots were blocked by incubation in a blocking buffer containing 5% (w/v) Marvel dried milk powder in PBS, pH 7.4 and

0.05% (v/v) Tween20 for 1 hour, at room temperature, before being immunoprobed with the appropriate primary antibody.

2.2.6.4.1. Immunoprobing of blots

Following blocking, the blots were incubated with the primary antibody in blocking buffer (5% (w/v) Marvel dried milk powder in PBS, pH 7.4) for 2 hours and at room temperature or, overnight at 4°C. Blots were then washed three times in 0.5% (v/v) PBS-Tween20, pH 7.4, for approximately five minutes each time, followed with a final 5 minute rinse in PBS, pH 7.4. The subsequent incubation with a species-specific secondary HRP-conjugated antibody, in the same blocking buffer, was performed for a further 2 hours at room temperature; on a rocking platform and in a plastic tray with a lid to minimise exposure to light and reduce evaporation. Following incubation, the membrane was washed three more times with 0.5% (v/v) PBS-Tween20, pH 7.4, for approximately five minutes each time, followed with a final 5 minute rinse in PBS, pH 7.4 to remove any residual milk and Tween20. The HRP conjugate of the secondary antibody was treated with the ECL chemiluminescence substrate according to the manufacturers' instructions (Amersham, Buckinghamshire, UK). Excess ECL substrate was blotted against lint-free tissue, before the membrane was covered with cling film and fixed, with tape, in a Kodak Biomax exposure cassette. The resulting light emission was detected by exposure to Kodak X-Omat chemiluminescence detection film (*Roche Diagnostics*, East Sussex, UK) for 1-20 minutes, depending on the dilutions of the antibodies and the intensity of the signal. The film was developed using 20% (v/v) LX-24 developer, fixed in 20% (v/v) FX-40 fixer and finally rinsed with distilled water before being air dried on the laboratory bench.

2.2.6.4.2. Membrane stripping

When further immunoprobing was required, the nitrocellulose membrane was soaked in PBS, pH 7.4, and then incubated in stripping buffer [100mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5mM Tris-HCL pH 6.7] at 50°C for 30 minutes with occasional shaking. The membrane was then washed twice in 0.5% (v/v) PBS-Tween20, pH 7.4, and re-blocked in a blocking buffer containing 1% (w/v) Marvel dried milk powder in PBS, pH 7.4 and 0.05% (v/v) Tween20 for 45 minutes.

2.2.6.5. Zymography

Gelatin and collagen zymography were carried out as previously described by Herron and co-workers [Herron *et al.,* 1986] but with the following adaptations: resolving gels were mixed with the following components, in order: 1ml of 5mg/ml of type I collagen solution in 20mM acetic acid (for collagen zymography) or, 1ml of 5mg/ml porcine gelatine in distilled water (for gelatin zymography), 3.1ml distilled water, 2.5ml of 1.5M Tris-HCl pH 8.8, 3.33ml of 29% acrylamide/1% N,N'-methylene bisacrylamide, 50µl of 10% ammonium persulphate and 10µl TEMED. The incorporation of SDS was found to cause precipitation of the collagen and so was not added to the resolving gel. Stacking gels were poured in the usual manner i.e. 0.65ml of 29% acrylamide/1% N,N'-methylene bisacrylamide, 3ml distilled water, 1.25ml 0.5M Tris-HCl, pH 6.8, 50µl of 10% SDS, 25µl of 10% ammonium persulphate, 5µl of TEMED. Samples containing MMPs were diluted 1:1 with loading buffer (0.1M Tris-HCl, pH 6.8, 50% glycerol, 0.4% bromophenol blue) and electrophoresed at 100V in standard Laemmli running buffer (24mM Tris-HCl, 192mM glycine, 3.47mM SDS, pH 8.3), and on ice in a chilled-cabinet (4°C) to avoiding overheating, taking approximately 4-5 hours. Following electrophoresis, the gels were washed twice, with shaking, for 30 minutes each time in 200ml of wash buffer consisting of 2.5% (v/v) Triton X-100, to remove the SDS and recover MMP activity. The gels were then placed in digestion buffer (100mM Tris-HCl, 5mM $CaCl_2$, 0.005% Brij-35, 1µM $ZnCl_2$, 0.001% NaN_3, pH 8.0) for 16-48 hours at 37°C. Gels were then stained with 0.2% Coomassie brilliant blue R-250 in 50% ethanol, 10% acetic acid for 2h and de-stained by microwaving for 15min (full power; 850W) in 3 changes of distilled water.

2.2.7. MOLECULAR ANALYSIS, DETERMINATION AND IDENTIFICATION

2.2.7.1. RNA extraction

RNA was isolated and purified using the SV Total RNA Isolation System (Promega, Southampton, UK) which involves four basic essential steps: the effective disruption of cells/tissues, denaturation of nucleoprotein complexes, inactivation of endogenous ribonuclease (RNase) activity and the removal of contaminating DNA and proteins. Samples were treated according to the manufacturers' instructions as followed: $1.5x10^3$ – $5x10^6$ cells were well mixed with 175µl of SV RNA Lysis Buffer by the pulsing of a pipette which ensured

clumps of cells were broken up- although additional shearing of the genomic DNA was achieved by passing the sample through multiple runs of a 20-gauge needle. 350µl of SV RNA Dilution Buffer was then added to the lysate and mixed by inversion, before being incubated at 70°C for exactly 3 minutes. Samples were then centrifuged at 13000g for 10 minutes, followed by the addition of 200µl of 95% ethanol. Further mixing was performed before the mixtures were transferred to the enclosed Spin Columns assemblies and centrifuged at 13000g for one minute. Samples were then washed with 600µl of SV RNA Wash Solution prior to another spin at 13000g for one minute. Immediately, 50µl/sample of a freshly made DNase incubation mixture (40µl core buffer, 5µl of 0.09M $MnCl_2$ and 5µl of DNase enzyme) was then added directly to the membrane within each spin basket and incubated for 15 minutes at 25°C. After this incubation, 200µl of SV DNase Stop Solution was added to the baskets prior centrifugation at 13000g, for one minute, followed by an initial wash with 600µl of SV RNA Wash Solution and another one minute spin at 13000g. A final wash with 250µl of the same buffer and another 13000g spin, for 2 minutes was also performed. The total RNA was eluted from the membrane by the addition of 100µl of nuclease-free water, before being pelleted with a one-minute 13000g spin. The purified RNA was stored at -70°C until required.

2.2.7.2. Determination of nucleic acid purity by UV spectrophotometry

Samples of nucleic acid were diluted 1/100 with distilled water before being loaded into quartz micro-cuvettes. Readings were then recorded at 260nm and 280nm using a DU-7 spectrophotometer (Beckman Instrument (UK) Ltd, High Wycombe, UK). The nucleic acid purity was determined using the ratio of A_{260nm} /A_{280nm} and normalised with the value of one A_{260} unit corresponding to 50µg/ml of RNA. Thus, the concentration of RNA was determined using the following formula:

$$[RNA]\ (\mu g/ml) = A_{260nm} \times 50 \times \text{dilution factor}$$

RNA solutions with a A_{260nm} /A_{280nm} ratio ≥1.7 were deemed free of phenol, and proteins, and hence suitable for further analyses.

2.2.7.3. Nucleic acid enrichment

Nucleic acid solutions with an A_{260} /A_{280} ratio ≤1.7 were mixed with equal volume of [phenol:chloroform:isoamyl alcohol] in the respective [25:24:1] ratio, before being centrifuged at 13000rpm (10000g) for 1 minute. The upper nucleic acid fraction was then transferred to a fresh tube and mixed with 0.25 volumes of 10M ammonium acetate and 2.5 volumes of ice cold ethanol, and stored at -20°C. Following overnight storage, the tube was then centrifuged at 13000rpm for 20 minutes, at 4°C, and the resulting pellet washed in 70% ethanol, air dried, before being resuspended in the appropriate buffer required.

2.2.7.4. Agarose gel electrophoresis of nucleic acids

0.5% (w/v) agarose gels were prepared by dissolving 1g of agarose (Bioline, London, UK) in 50ml of 1x Tris-Acetate-EDTA buffer (TAE: 40mM Tris, 0.114% (v/v) glacial acetic acid and 1mM EDTA) by heating the solution, twice, for 1 minute each time in a microwave (full power; 850W). Once cooled, 5µl of ethidium bromide was added to the solution to a final concentration of 0.5mg/ml and the gels cast in a Bio-Rad DNA-Sub electrophoresis tray (Bio-Rad, Hertfordshire, UK). The gel was allowed to set for 50 minutes, at room temperature, on a level laboratory bench. Samples were mixed with 10x DNA loading buffer (20% (v/v) ficoll 400, 100mM EDTA, 1% (w/v) SDS, 0.25% (w/v) bromophenol blue, 0.25% xylene cyanol) before being loaded into the into the sample wells. Electrophoresis was performed at 90V for 80 minutes (or, until the bromophenol blue dye front had migrated approximately ¾ of the length of the gel) in 1x TAE running buffer. Confirmation was achieved by viewing the gel under UV light to verify loading and nucleic acid integrity

2.2.7.5. Denaturing agarose gel electrophoresis of RNA

A minimum of 20µg of RNA was electrophoresed on a 1.2% (w/v) ultra pure agarose/1x morpho-linepropane-sulfonic acid (MOPS)/ 2% (v/v) formaldehyde gel. RNA samples were prepared by combining 20µg of RNA with 25µl of RNA sample buffer [containing 80% w/v deionised formamide, 8% (v/v) formaldehyde, 50µl 10x MOPS buffer, 38µg/ml ethidium bromide, 3.5% (v/v) Ficoll, 10mM EDTA, 0.05% (w/v) bromophenol blue] and heated at 65°C for 15 minutes. The treated RNA was then pipetted into the sample wells of the gel, before being

electrophoresis at 90V for 90 minutes or, until the bromophenol blue dye front had migrated approximately ¾ of the length of the gel. Confirmation was achieved by viewing the gel under UV light to verify loading and RNA integrity by the presence of intact 28S and 18S ribosomal bands at 4.5 and 1.9kb respectively with an intensity ratio of 2:1.

2.2.7.6. Northern blotting

Resolved RNA, following electrophoresis, was transferred to Hybond-N nylon membranes (Amersham, Buckinghamshire, UK) using an improvised capillary blotting system: the transfer apparatus consisted of a glass plate placed over a plastic tray which served as a reservoir of 20% SCC buffer (3M NaCl, 0.3 M sodium citrate; pH 7.0). A sheet of Whatman 3MM paper was manipulated into a wick, before being placed onto the glass plate in such a way that the overhanging edges were completely immersed in the reservoir below. The RNA sample gel was then inverted prior being placed onto the sheet-wick. To prevent any short-circuiting and minimise evaporation of the buffer, the entire perimeter of the gel was covered in Parafilm (SLS, Nottingham, UK). The Hybond membrane was then placed across the upper surface of the gel; air bubbles, if any, were removed by rolling a glass rod over the membrane. The additional overlay of several sheets of Whatman 3MM filter paper and, approximately, a 12cm stack of paper towels were placed over the membrane. A 1Kg weight was then applied on top of the towels and the transfer allowed to proceed overnight at room temperature. Once the transfer was complete, the apparatus was disassembled and the membrane marked with pencil to identify the position of the sample wells and the ribosomal bands. The RNA was fixed to the filter by UV irradiation (70mJ/cm^2) using a UV cross-linker (Amersham, Little Chalfont, UK).

2.2.7.6.1. Priming of cDNA probes

The specific primed DNA probes were labelled from the appropriate DNA sequences kindly supplied in a suitable vector by Dr T. Johnson (Sheffield Kidney Institute, Northern General Hospital, Sheffield, UK). The purified cDNA (12.5ng) was random-primed with ^{32}P-labeled dCTP (Redivue, Amersham, UK) using the Prime-a-Gene™ system (Promega, Southampton, UK) according to the manufacturers' instructions. Excess unincorporated nucleotides were

removed using Sephadex G50 Nick Columns™ (Amersham Pharmacia, Buckinghamshire, UK) according to the manufacturer's instructions. Furthermore, the random primed cDNA probes were denatured by boiling for 3 minutes, then immediately placed onto ice to prevent re-annealing prior to addition to the hybridisation solution.

2.2.7.6.2. Hybridisation of cDNA probes and autoradiography

Membranes were pre-hybridised in ExpressHyb™ hybridisation solution (Clontech, Cowley, UK) at a probe specific temperature, for 1 hour, in a Techne Hybridser HB-1D dryer oven (Jencons-PLS, East Sussex, UK). Hybridisation was performed under the same conditions but with the addition of the labelled probe to 1x 10^6 cpm/ml for 18 hours. The filters were then subjected to multiple washes with 2x and 0.2x saline-sodium phosphate-EDTA, 0.25% SDS buffers at temperatures ranging from room temperature up to 65 °C for a total of 80 minutes, after which they were then exposed to Kodak XOMAT AR/LS film for up to 7 days, at -80°C, in intensifying screens. Loading was corrected by reference to the optical density of the previously measured ethidium bromide-stained 18S rRNA as well as the housekeeping gene, cyclophillin. Determination of the transcript size was carried out by reference to RNA molecular weight markers (Promega, Southampton, UK) and further confirmed by visual comparison to the ribosomal RNA subunits. Nylon membranes were routinely stripped by boiling in 0.1% (w/v) SDS for at least 30 minutes for further blots. Completed Northern blots were quantified using Phoretix 1D image analysis software (NonLinear Dynamics, Newcastle-upon-Tyne, UK).

2.2.7.7 PCR analysis of TG-2 and TG-1 for MEF cells

Total RNA was extracted from 2 x 10^7 cells using the RNeasy minikit from Qiagen (Crawley, UK). Reverse transcription (RT)-PCRs were performed with the RT-PCR One Step System (Life Technologies, Paisley, UK), using 200ng of total RNA, according to the manufacturer's instructions. The primers used for the amplification of TG-2 were TG30 (5'GACAACAACTATGGGGATGGT3') and TG9B (5'ATCATCTCGCTCTTGTTCGTC3'). The primers used for the amplification of TG-1 were MTG1F (5'ACCACCACAGTGCTCCGATG3') and MTG1R (CCACACGTGGAAGTTCCAAAC3'). The following PCR program was

used in all cases: 42°C for 30 min and 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 57°C for 30 s, and 70°C for 30 s. PCR products were resolved on a 1.6% agarose gel and stained with ethidium bromide.

2.2.8. STATISTICAL ANALYSIS

Data sets were tested for normality and subsequent tests of comparisons were chosen appropriately. For simple paired comparisons, the Mann-Whitney or student *t*-test, using the Minitab or Sigma Stats software packages, were utilised and expressed as mean ±SD. For normally distributed multiple data comparisons, a one-way ANOVA test was utilised and expressed as mean ±SD. Whenever the statistical significance between control and treated samples were evaluated at a 95% confidence level ($p<0.05$), the data set would be considered to be statistically significant and represented with a * on the bars.

CHAPTER 3:
PHYSICAL CHARACTERISATION

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3.1. INTRODUCTION

Collagen is frequently chosen as the scaffold for medical implants as it is not only the main component of the ECM but, also biodegradable, has low antigenicity and good cell-binding properties [Lee *et al.,* 2001; Weinberg and Bell, 1986; Bell *et al.,* 1981]. However, it is essential that the physical property of the applied material be similar to the tissue that is being replaced and it must also be able to maintain the desired *in situ* characteristics e.g. elasticity for expansion/contraction as vessels or, withstand stress-strain forces when applied as a tendon or bone substitutes [Kato and Silver, 1990]. Unfortunately, native collagen once processed and reformed suffers reduced mechanical strength, experiences rapid degradation *in vivo* and, as a consequence, becomes ineffective in the management of infected and tissue-damaged sites [Angele *et al.,* 2004; Goo *et al.,* 2003; Koob and Hernandez, 2002; Koob *et al.,* 2001; Friess, 1998].

As such, various approaches to cross-linking the collagen have been used to improve its biological stability, ease of handling and its mechanical characteristics for implant applications [Coombes *et al.,* 2002; Jorge-Herrero *et al.*, 1999; Dunn *et al.*, 1993; Kato *et al.,* 1991] with many investigators also showing that increasing cross-linking within the collagenous material results in enhanced thermal stability and resistance to degradation [Bann *et al.*, 2000; Goo *et al.*, 2003]. It has thus been demonstrated over the years that the physical properties of cross-linked collagen are not only highly influenced by the number of cross-links present in the material but, also, by the treatment employed in the cross-linking reaction [Charulatha and Rajaram, 2003]. In short, the ideal biomaterial must meet several requisites; firstly, the mechanical properties of the construct must be equivalent to the target application, the material must be biocompatible, eliciting little if any foreign body or antigenic response and allow cell attachment, replication and neo-matrix synthesis and assembly. Ultimately, the cytotoxicity of the material, residues chemicals and/or byproducts must be eliminated [Koob *et al.*, 2001].

Previous work within our laboratory with commercial tissue transglutaminase demonstrated the presence of a wide range of protein contaminants as well as a frequent deviation of activity from batch to batch. Hence, the initial aim of

this chapter is to define and perform a quality assurance/control of the materials to be used in the experiments- with concentration, dilution factors and costing of the other raw materials considered and assessed. The focus will then be to determine whether the TG enzymes are capable of cross-linking native collagen and to identify the optimum conditions for such a reaction to occur. Furthermore, the aims will be to characterise any physical changes to the native collagen that result from the cross-linking modification, namely: the mechanical strength and structure, rheological properties, antioxidant ability and differences in the susceptibility of the modified collagen to proteolytic attack and/or degradation.

3.2. RESULTS

3.2.1. Quality control: Transglutaminase

The tissue transglutaminase use in this investigation was obtained 'in-house' (purified form guinea pig liver) using a combination of anion exchange, gel filtration and affinity chromatography as previously described by LeBlanc and colleagues with a few modifications [LeBlanc *et al.*, 1999] and is referred as "tTG" throughout. The protein and enzyme activity profiles of the aforementioned column steps can be seen in Figures 3.2.1 A, B and C, respectively, following the consecutive loading of pooled high specific activity fractions. Although tTG purification is a relative simple process according to previous investigators [Folk and Chung, 1985; Folk and Cole, 1966], the adaptation to those procedures within our laboratory gave rise to certain difficulties: during the GTP-affinity purification step, it was found that fractions from the affinity step were not compatible with the hydroxamate activity assay nor the Lowry protein assays due to the high concentration of GTP. Hence tTG activity was determined using the BTC assay and protein concentration monitored by the absorbance of the fractions at 280nm, as seen in Figure 3.2.1C. Further confirmation of the presence and purity of the isolated tTG was achieved through SDS-PAGE electrophoresis on the selected post-GTP fractions, as shown in Figure 3.2.2, that confirmed a sole protein at ~78kDa.

As mentioned early, the use of an 'in-house' purified enzyme was deemed necessary due to the economical costs of the investigation but, more importantly because of the poor quality of the commercially available enzyme product. Figure 3.2.3A shows a merged SDS-PAGE gel of samples from each stage of the employed purification process as well as that of the commercially available guinea pig liver tTG enzyme (gpl-tTG). It can be seen that a successful isolation, purification and concentration profile of the enzyme is achieved during the consecutive steps in this protocol; from the crude liver homogenate to the highly purified tTG post-dialysis. Furthermore, the lane profile of the commercial gpl-tTG can be seen to contain a degree of other protein contaminants. Further confirmation can be seen in Figure 3.2.3B, a western blot of the same samples with a monoclonal antibody specific to tTG (CUB7402), clearly highlighting the presence of gpl-tTG degradation products of the main enzyme.

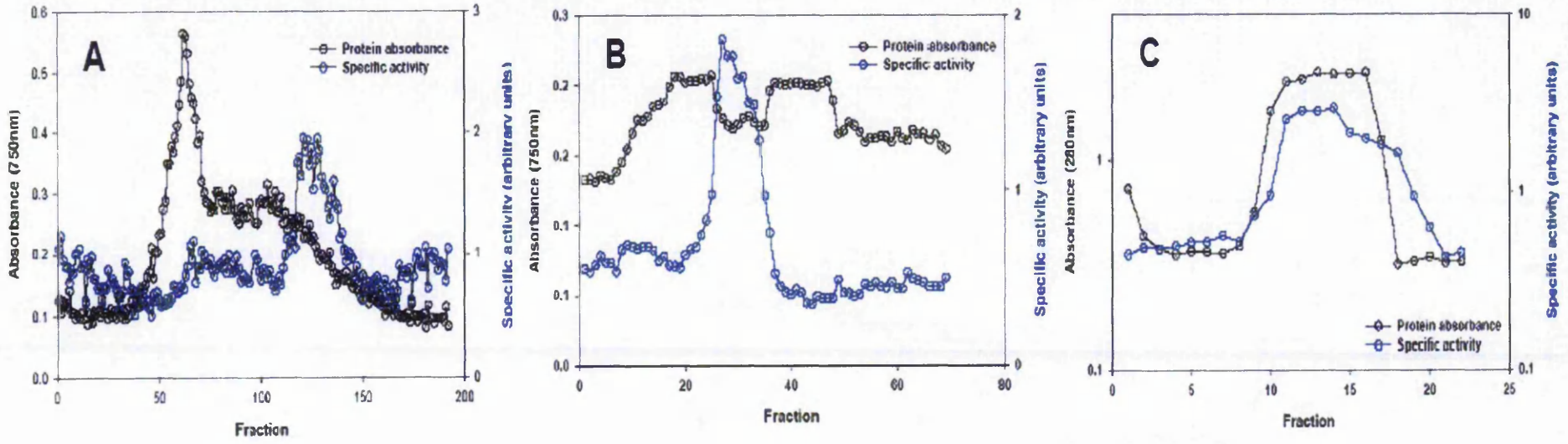

Figure 3.2.1. Protein and enzyme activity curves during tTG isolation and purification. Profiles from the consecutive loading of pooled high specific activity fractions during ion exchange chromatography using Q-Sepharose Fast Flow with a linear gradient of 0-720mM NaCl at a flow rate of 15ml/min (**A**); gel filtration with a Sephracryl S-200 Bio-Gel A-0.5m (fine mesh) packed column, at a flow rate of 2ml/min (**B**); GTP-agarose affinity chromatography at a flow rate of 1.5ml/min (**C**). Fractions during the ion exchange and gel filtration chromatography steps were assayed for protein concentration and tTG activity using the Lowry (measured at 750nm) and hydroxamate assays (measured at 492nm), respectively. For the GTP-agarose affinity purification step, the enzyme activity was determined by the BTC assay (measured at 450nm) and protein concentration by the absorbance of fractions at 280nm. High specific activity fractions were chosen for further analysis by SDS-PAGE and western blotting.

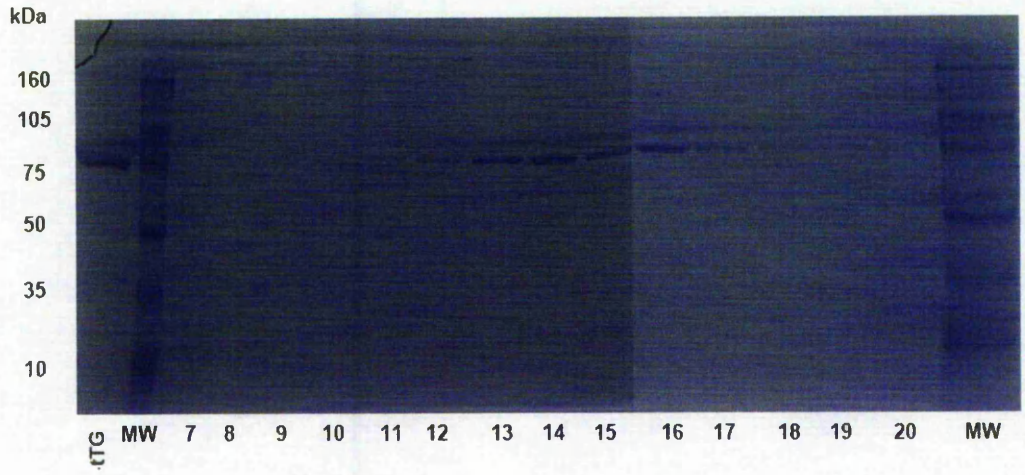

Figure 3.2.2. SDS-PAGE of fractions from GTP-agarose affinity chromatography purification. 6-12µg of selected protein samples (fraction numbers 7-20) from the affinity chromatography step were electrophoresed on a 10% SDS-PAGE gel at 150V for 1.5 hours. Resolved proteins were stained with 0.1% (w/v) Coomassie Brilliant blue (in 40% (v/v) methanol, 10% (v/v) acetic acid and 50% (v/v) dH_20) and then destained using a destain solution (10% (v/v) methanol, 10% acetic acid, 80% (v/v) dH_20). Lane number corresponds to fraction during affinity chromatography. MW, molecular weight markers (Promega, Southampton, UK); TG, tTG standard.

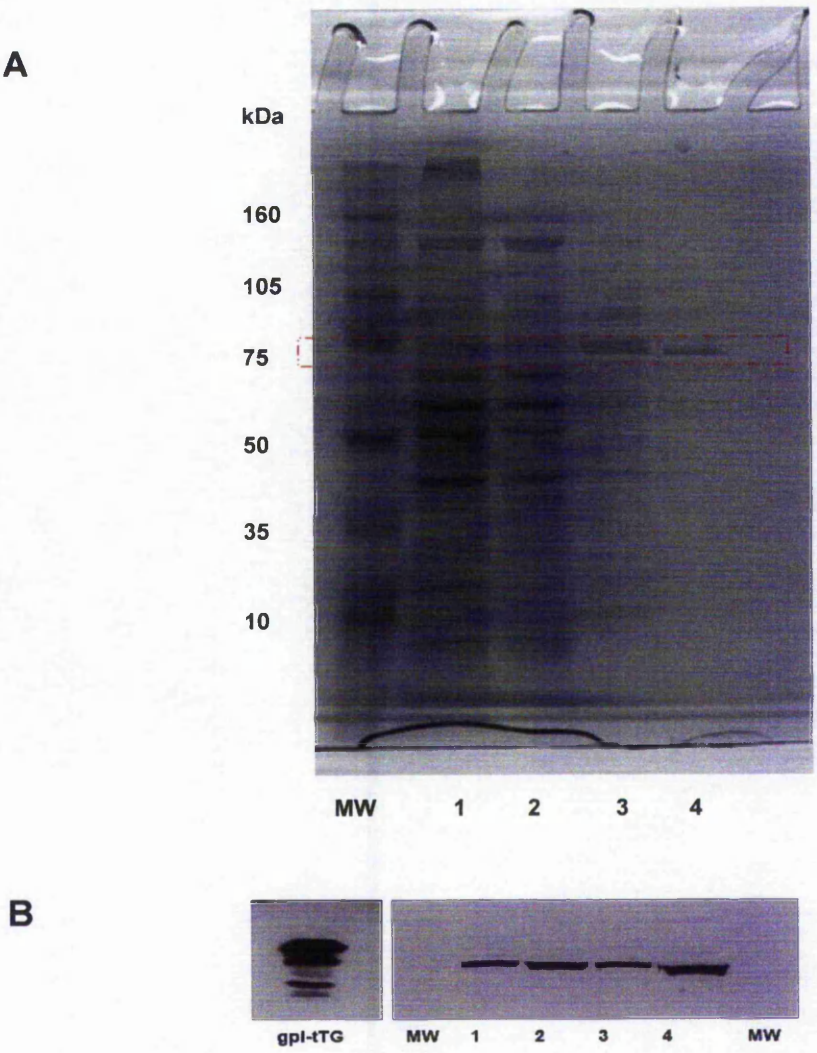

Figure 3.2.3. SDS-PAGE and western blot of pooled fractions during tTG isolation and purification. 6-12µg of selected pooled protein fractions from each step of the tTG purification were electrophoresed on a 10% SDS-PAGE gel at 150V for 1.5 hours (**A**). Resolved proteins were stained with 0.1% (w/v) Coomassie Brilliant blue (in 40% (v/v) methanol, 10% (v/v) acetic acid and 50% (v/v) dH_20) and then destained using a destain solution (10% (v/v) methanol, 10% acetic acid, 80% (v/v) dH_20). Lane 1, crude liver homogenate; lane 2, post ion exchange chromatography; lane 3, post gel filtration chromatography; lane 4, post GTP-affinity chromatography and dialysis (5kDa MW cut-off point). MW, molecular weight markers (Promega, Southampton, UK); gpl-tTG, commercial guinea pig liver tTG (Sigma, Poole, UK). Red box highlights tTG protein band. (**B**) Western blot for tTG of the same samples using a monoclonal anti-tTG antibody (CUB7402; Neomarkers, Fremont, USA; at 1:1000 dilution).

Samples from the 'in-house' purified preparation shows only a single band with the CUB7402 MAb suggesting that only the highly purified tTG enzyme exists.

The microbial transglutaminase (mTG) employed in this investigation was obtained from the commercially available Actima™ WM product (Ajinomoto Corporation Inc., Japan). Although mTG is the only protein present in this preparation, further purification is required to remove the maltodextrin which contributes to 99% of the total final product. Hence, ion exchange chromatography was used as the single concentrating and cleaning step; of which a single peak corresponding to the specific activity of the enzyme can be deduced from the protein and enzyme profiles as shown in Figure 3.2.4A. Validation of the purity of the mTG was achieved through SDS-PAGE characterisation of the pooled fractions and confirmed a sole protein at ~37Da, as shown in Figure 3.2.4B. However, some 'smearing" is evident and most likely due to mTG breakdown products as a result of long-term storage. Western blotting of the same sample with a polyclonal antibody specific to (*Sv. mobaraensis*) mTG, as seen in Figure 3.2.4C, confirms the presence of mTG as well as the presence of the aforementioned breakdown byproducts.

Although the activities of the enzymes were constantly monitored throughout the purification process, the incorporation of [^{14}C]-putrescine into N,N'-dimethylcasein assay was deemed necessary due to its higher accuracy and sensitivity and for quality control purposes. As presented in Figure 3.2.5, the activities of gpl-tTG, tTG and mTG were compared against each other and based on the equal loading (80µg/ml) of each enzyme. It can be seen that mTG has significantly greater activity (1600nmol putrescine incorporated/hr/mg protein); approximately 1.5 times greater than the in-house purified tTG enzyme ($p<0.05$). The commercially purified gpl-tTG has the lowest activity at around 600nmol putrescine incorporated/hr/mg protein. The most likely reason being due to the presence of other protein contaminants or, a result of enzyme degradation following prolonged storage or from a relatively poor purification procedure- as discussed earlier. Hence, all experiments throughout this investigation involved the higher activity and greater quality in-house purified tTG. Sufficiently large batches of the enzymes were purified each time to maintain enzyme-activity consistency and prevent batch-to-batch variations between experiments.

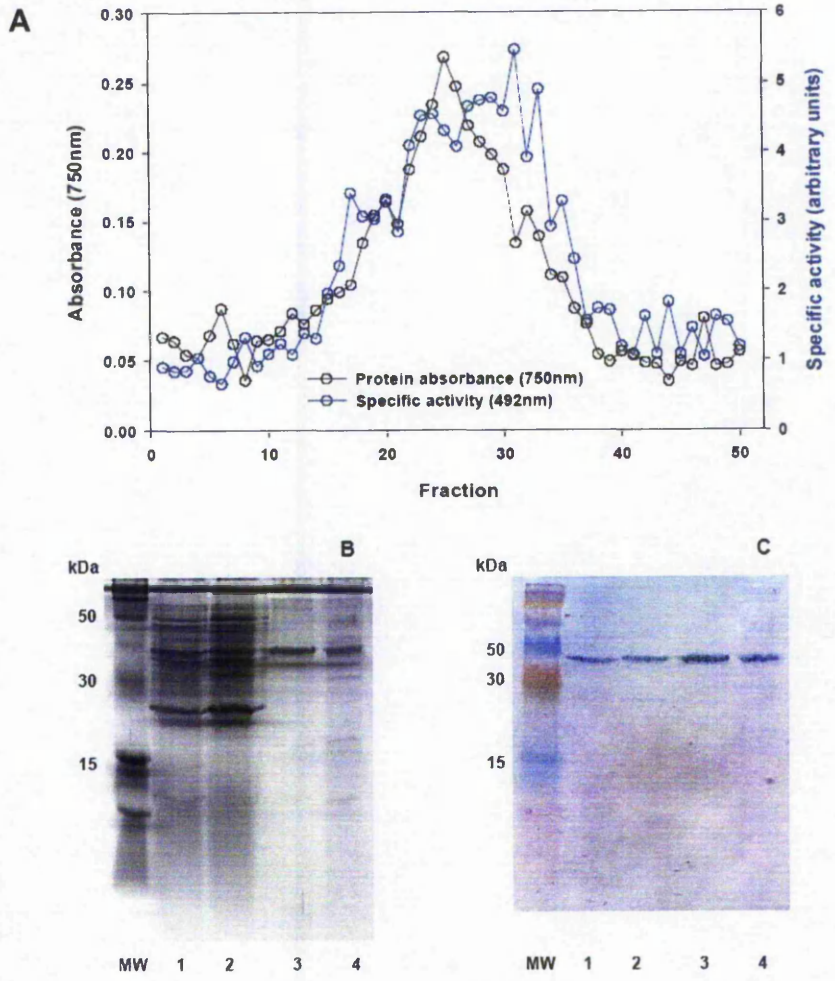

Figure 3.2.4. Analysis during mTG purification and enrichment. Protein and enzyme activity profiles for fractions during ion exchange chromatography using SP-Sepharose Fast Flow column with a single linear gradient of 0-1000mM NaCl set-up running at 5ml/min (**A**). High specific activity fractions were chosen for further analysis by SDS-PAGE and western blotting: 6-12µg of the pooled protein fractions from the ion exchange chromatography step were electrophoresed on a 12% SDS-PAGE gel at 150V for 1.5 hours (**B**). Resolved proteins were stained with 0.1% (w/v) Coomassie Brilliant blue and then destained using a destain solution as described in the Methods. MW, molecular weight markers (Promega, Southampton, UK); lane 1, supernatant from ion exchange chromatography; lane 2, flow-through from ion exchange chromatography; lane 3, pooled fractions from ion exchange chromatography.; lane 4, pooled fractions from ion exchange chromatography (**C**) Western blot for mTG of the same samples using a polyclonal anti-mTG antibody (anti-*Streptomyces mobaraensis*; N-Zyme Biotech, at 1:1000 dilution).

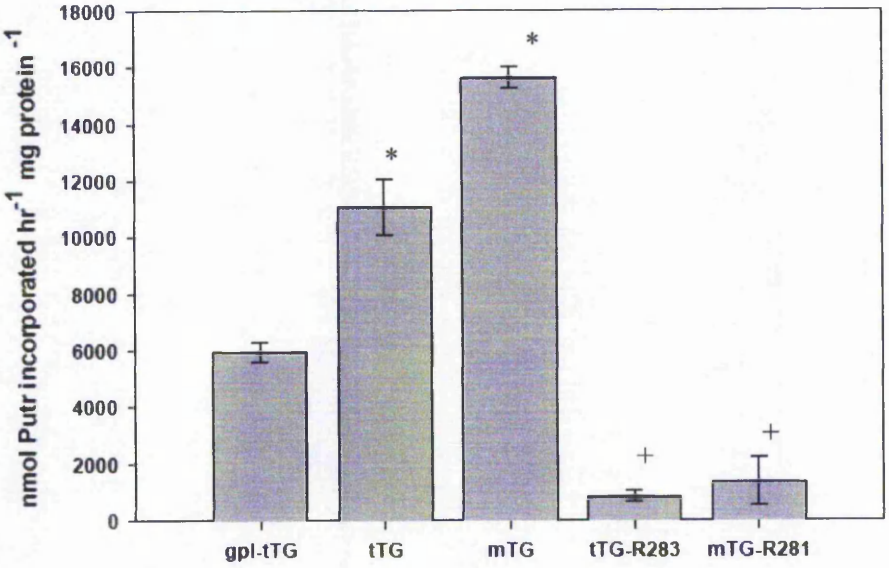

Figure 3.2.5. Specific activity of the transglutaminases. The enzymatic activity of the different transglutaminases (80µg/ml) was determined using the [^{14}-C]-putrescine assay. tTG and mTG were inactivated using the site-directed inhibitors, R283 and R281, respectively (500µM; 6 hours, 4ºC). Control samples were also incubated at 4ºC for 6 hours. Abbreviations: gpl-tTG, commercial guinea pig liver tTG (Sigma); tTG, tissue transglutaminase; mTG, microbial transglutaminase; tTG-R283, tTG inactivated with R283 inhibitor; mTG-R281, mTG inactivated with R281 inhibitor. Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments. Specific activities of the individual enzymes were compared to the activity of gpl-tTG and values corresponding to $p<0.05$ when compared with controls are represented with a *. Results obtained in the presence of inhibitors were compared against the results in its absence and values corresponding to $p<0.05$ are represented with a +.

Inhibition of tTG and mTG was achieved (via irreversible inactivation of the active site cysteine) using 500μM of either R283 or R281, respectively, achieving >95% inhibition as also shown in the inhibition assay in Figure 3.2.5. As a consequence, this concentration of inhibitor was used during the investigation as negative controls for experiments.

3.2.2. Quality control: Collagen

Although some experiments were performed using a commercially sourced collagen, the ongoing financial constraints, batch-to-batch variation and large demand of the material consequently led to an in-house purification procedure for the sourcing of the collagen. Throughout this investigation, the type I collagen was sourced from calf tendons via an acid precipitation protocol. Figure 3.2.6A shows the SDS-PAGE gel of isolated calf tendon, rat tail and commercial calf skin type I collagen. The distinct α1 and α2 chains can be identified (at approximately 120kDa and 140kDa). It can also be noted that the commercial calf skin collagen contains traces of impurities as highlighted by the presence of a contaminating protein ladder. Further confirmation of the collagen was achieved via western blotting using a monoclonal antibody specific to type I collagen as shown in Figure 3.2.6B.

3.2.3. Cross-linking of collagen by transglutaminases

Native collagen type I was treated with both tTG and mTG, separately, in order to catalyse the formation of ε-(γ-glutamyl)lysine cross-linking. The extent of cross-linking for each of the TG treatments is shown in Table 3.2.1. Treatment of collagen with increasing concentrations of TG led to a corresponding increase in the amount of ε-(γ-glutamyl)lysine bonds present- with up to 1mol of cross-link per mol of collagen monomer. Treatment with mTG gave a much greater increase (almost two-fold) in the amount of isopeptide formed for the equivalent protein concentration of transglutaminase used. However, it can be suggested that the increased specific activity of the mTG (as outlined in section 3.2.1) probably accounts for the differences noted.

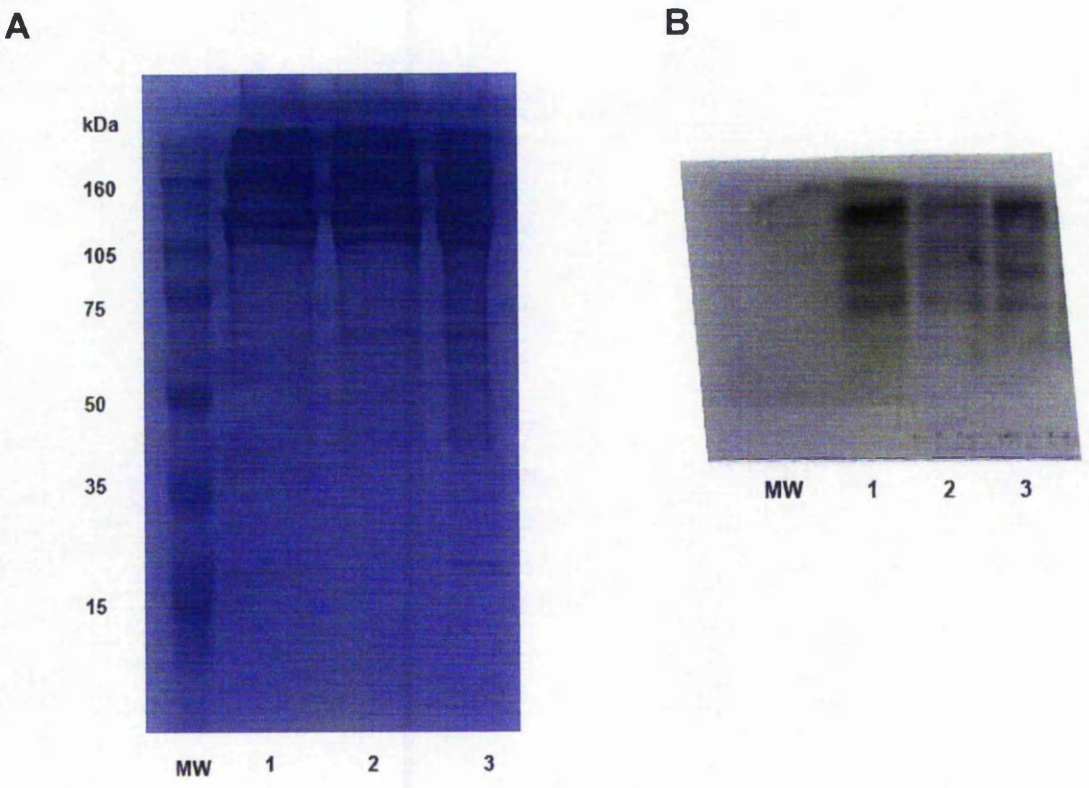

Figure 3.2.6. SDS-PAGE and western blot of collagen type I. Collagen samples (6-12µg of protein) were fractionated on a 5% SDS-PAGE gel at 150V for 1.5 hours as described in the Methods (**A**). Resolved proteins were stained with 0.1% (w/v) Coomassie Brilliant blue as described in the Methods. MW, molecular weight markers (Promega, Southampton, UK); lane 1, collagen type I from calf tendon; lane 2; collagen type I from rat tail tendon; lane 3, commercial collagen type I from calf skin (Sigma, Poole, UK). (**B**) Western blot for collagen type I using a monoclonal anti-collagen type I antibody (C2546; Sigma, Poole, UK; at 1:1000 dilution). MW, molecular weight markers; lane 1, collagen type I from bovine tendon; lane 2; collagen type I from rat tail tendon; lane 3, commercial collagen type I from calf skin.

Sample	TG concentration (µg/ml)[$]	nmol of cross-link/ mg protein sample	± relative change to native collagen[*]	mol cross-link/mol of collagen[+]
Collagen	–	0.16	–	0.02
Coll-tTG	50	1.09	6.81	0.13
Coll-tTG	100	2.40	15.00	0.29
Coll-tTG	200	4.60	28.75	0.55
Coll-tTG	500	5.40	33.75	0.65
Coll-tTG	1000	8.90	55.63	1.07
Coll-mTG	10	0.90	5.63	0.11
Coll-mTG	50	2.00	12.5	0.24
Coll-mTG	200	4.90	30.63	0.59
Coll-mTG	500	8.40	52.50	1.00

[+] M_w collagen: 120kD; [*] native collagen = 0.16nmol crosslink; [$] TG activity: tTG = 11500-13000 Units/mg; mTG = 16000-25000 Units/mg

Table 3.2.1. Measurement of ε-(γ-glutamyl)lysine in TG-cross-linked collagen. Native collagen was treated with differing concentrations of both tTG and mTG (enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg) for 8 hours. Samples were then proteolytically digested as described in the Methods prior to being analysed on to a Dionex DC-4A resin column and Pharmacia Alpha Plus amino acid analyser for ε(γ-glutamyl)lysine crosslink. Sample values are based on mg (hydrolysed) protein. Quantification of the dipeptide was determined by standard addition. Values determined from a single batch of samples.

3.2.4. Residual transglutaminase entrapped in cross-linked matrices

To ensure that no residual TG remained in the cross-linked matrices and, also, to confirm the effectiveness of the 'washing steps' employed during this investigation, the presence of any potential entrapped TG was monitored by SDS-PAGE and western blotting as shown in Figures 3.2.7 (A-D). Following treatment of native collagen with both tTG and mTG, it can be seen that two consecutive washes with PBS are required to remove any entrapped tTG; whereas only a single wash-step was sufficient to remove mTG from within the collagen matrix as shown by the presence/absence of the TG band in the gels in Figures 3.2.7 A and C respectively. Further confirmation is also provided by the presence/absence of the corresponding bands in the western blots for tTG and mTG as seen in Figures 3.2.7 B and D respectively.

3.2.5. Atomic force microscopy of native and TG-cross-linked collagen

Native collagen type I treated with 250µg/ml of either tTG or mTG, was analysed using atomic force microscopy (AFM) under 'tapping mode' settings. Images were captured using the in-built (Veeco Dimension 3100) digital camera, analysed with the Nanoscope software platform and shown in Figure 3.2.8. The untreated collagen (Figure 3.2.8A) shows a typical matted arrangement of straight fibrils, with diameters of between 100-300nm and the typical banding pattern (D-period) of 67nm. Tissue TG-treated collagen (Figure 3.2.8B) shows only a minor difference to the normal collagen structure; the fibrils appear very similar to untreated collagen with respect to diameter and arrangement. However, the D-period of tTG-treated collagen is reduced to approximately half that of untreated collagen. Interestingly, the mTG-treated collagen results in a total breakdown of the normal structure as seen in Figure 3.2.8C. The fibrils are much narrower than seen in the untreated collagen and tend to adopt a parallel (or anti-parallel- it is impossible to tell) alignment relative to each other with a dominant helical-twist overlay. Moreover, no noticeable banding (D-period) exists with the individual fibrils.

3.2.6. Rheological monitoring of native and TG-cross-linked collagen

Figures 3.2.9 A and B show the time course variation of the elastic (G') and viscous (G'') moduli, respectively, for native and mTG-treated collagen. It can

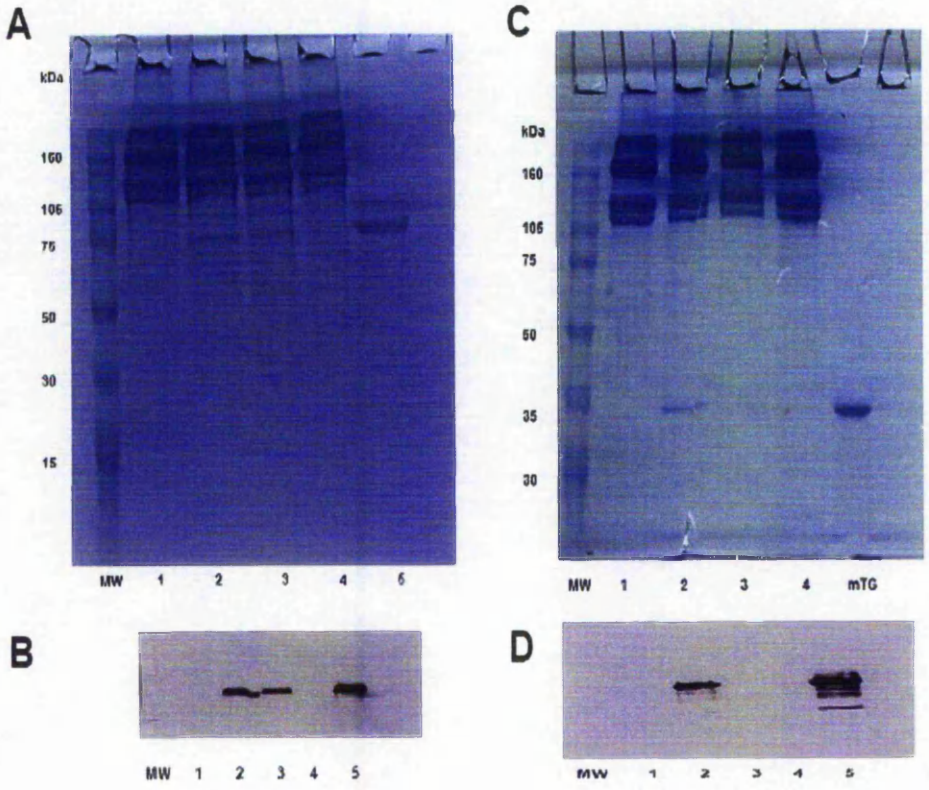

Figure 3.2.7. SDS-PAGE and western blot for residual TG enzyme entrapped within matrices. Native collagen was treated with 250µg/ml of TG (enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg) and then subjected to consecutive washing steps with PBS, pH 7.4. before being fractionated on a 5% SDS-PAGE gel at 150V for 1.5 hours. The resolved proteins from (**A**) tTG treatment and (**C**) mTG treatment were stained with 0.1% (w/v) Coomassie Brilliant blue as described in the Methods. MW, molecular weight markers (Promega, Southampton, UK); lane 1, native collagen type I from bovine tendon; lane 2; collagen treated with 250µg/ml of TG; lane 3, cross-linked sample subjected to 1 wash with PBS; lane 4, cross-linked sample subjected to 2 washes with PBS; lane 5, TG standard (gpl-tTG or mTG). (**B**) and (**D**) Western blots for tTG and mTG, respectively, using a monoclonal anti-tTG antibody (CUB7402; Neomarkers, Fremont, USA; at 1:1000 dilution) or a polyclonal anti-mTG antibody (anti-*Steptomyces mobaraensis*; N-Zyme Biotech, Darmstadt, Germany; at 1:1000 dilution). MW, molecular weight markers; lane 1, native collagen type I from bovine tendon; lane 2; collagen treated with 250µg/ml of TG; lane 3, cross-linked sample subjected to 1 wash with PBS; lane 4, cross-linked sample subjected to 2 washes with PBS; lane 5, TG standard (gpl-tTG or mTG).

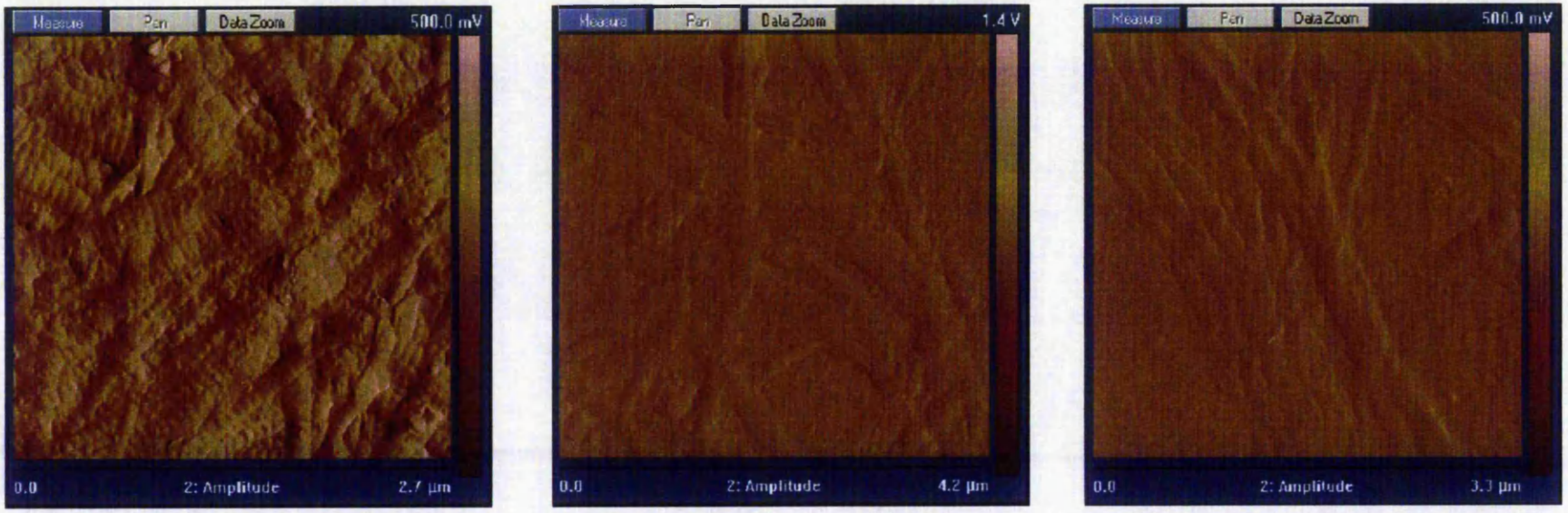

Figure 3.2.8. Atomic force microscopy of TG-cross-linked collagen. Native collagen (**A**); collagen treated with 250ug/ml of tTG (**B**); and collagen treated with 250ug/ml of mTG (**C**) were analysed using A Dimension 3100 atomic force microscope (Veeco Instruments Ltd., Cambridge, UK), utilising 'tapping mode' settings with a drive frequency close to the resonance frequency of the cantilevers as described in the Methods. Enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg. Imaging was conducted simultaneously in the height mode and phase mode. The ratio of the set point value to the free amplitude of the cantilever was maintained at approximately 1.0. Scan size was set at 0.8-8µm (scan rate, 1Hz). The drive amplitude of the cantilever was set to a root mean square (RMS) value of 18-30nm. All samples were viewed and imaged at room temperature. Captured images were achieved using the in-built digital camera and further analysed with the Nanoscope software platform.

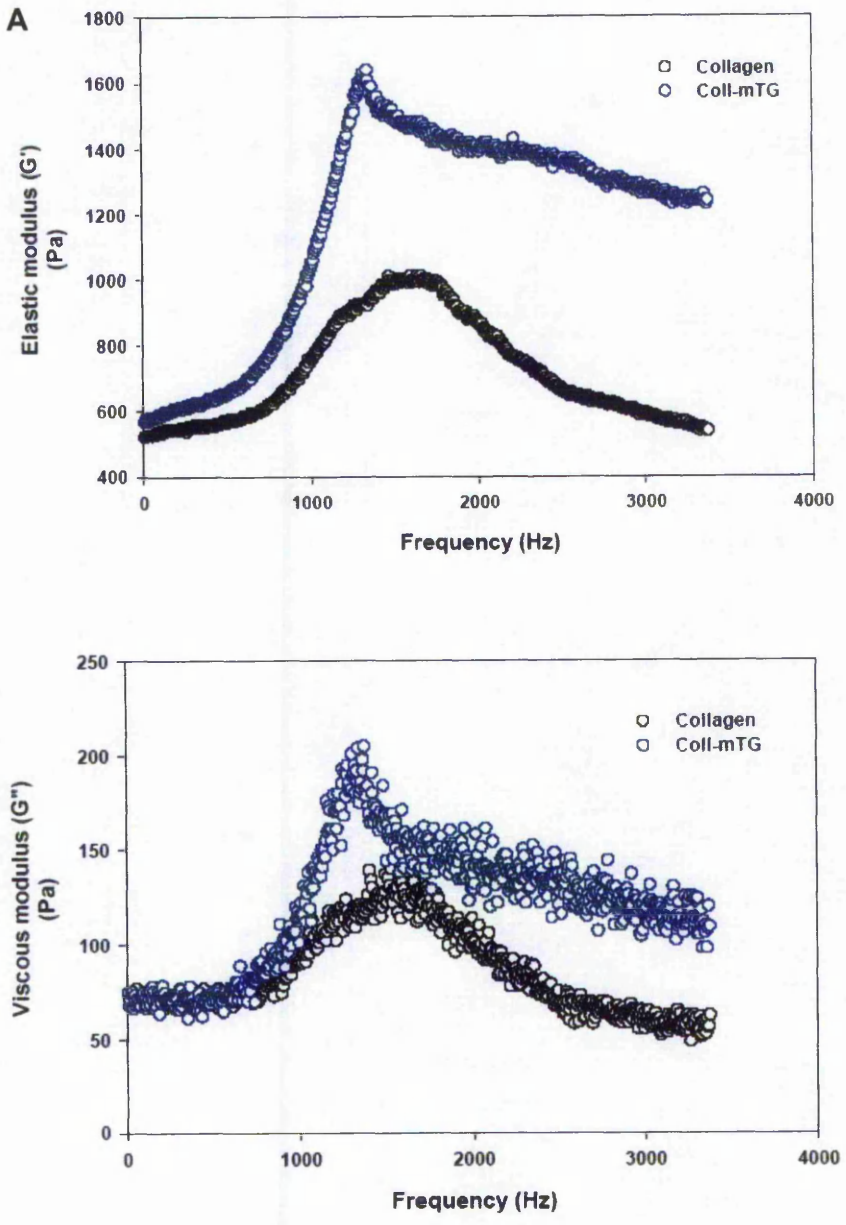

Figure 3.2.9. Rheological properties of native and mTG-treated collagen. The elastic (G') modulus (**A**); and the viscous (G") modulus (**B**) for native and mTG-treated collagen (250µg/ml) were determined using a Bohlin C-VOR KTB30 rheometer fitted with a cylindrical Couette geometry set-up and 2cm diameter serrated plates as described in the Methods. Enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg. The plate-gap dislocation distance was set at 0.8mm with sample conditions maintained at 37°C.

be seen that sufficiently large changes and deviations occur for both the elastic and viscous moduli of the mTG-treated collagen, as opposed to the native collagen, indicating a stronger and, possibly, more stable gel formation. Furthermore, a shorter time period is required for the gelation process for mTG-treated collagen than the native variant. The variation of *in situ* fibrillogenesis of the native and mTG-treated collagen, as monitored by the elastic (G') and viscous (G'') moduli, can be seen in Figure 3.2.10. It can been deduced that gelation occurs almost immediately for the mTG-treated collagen as opposed to the delayed response (~1500 seconds) experienced by the native collagen. Additionally, a more linear gradient response is demonstrated by the elastic and viscous moduli of the mTG-treated collagen, with greater differences between the two factors, suggesting that a "firmer" gel is formed.

Addition of both tTG and mTG to neutralised type I collagen, *in vitro*, similarly increased the rate of fibril formation as measured by the turbidity in a dose dependent manner as shown by Figures 3.2.11 A and B. Interestingly, a significant decrease in the final level of fibril formation was noted with both the TG-treatments ($p<0.05$). A comparable dose-dependent effect was also noted for the treatment of neutralised type III collagen, as seen in Figures 3.2.12 A and B, of which mTG treatment produced a more significant increase in the rate of fibril formation than tTG ($p<0.05$). However, in contrast to the effect observed for type I collagen, an increase in the final level of fibril formation was noted with both the TG-treatments. Inhibition of the TG activity using the R281 inhibitor confirmed that these effects were based solely on the TG activity.

3.2.7. Antioxidant properties of native and mTG-treated collagen

Native and mTG-treated collagen were treated using the colorimetric $ABTS^{\bullet+}$ free-radical scavenging assay in order to determine their antioxidant abilities. It can be seen in Figure 3.2.13A that the specific absorbance, $(Abs_{time}/Abs_{100\%})^{-1}$, of mTG-treated collagen is significantly greater than that when compared to the native collagen and this capability prevails throughout the entire time course of the assay ($p<0.05$). This suggests that mTG-treated collagen possesses a much greater free-radical scavenging ability. Inhibition of the mTG activity using the R281 inhibitor confirmed that these effects were

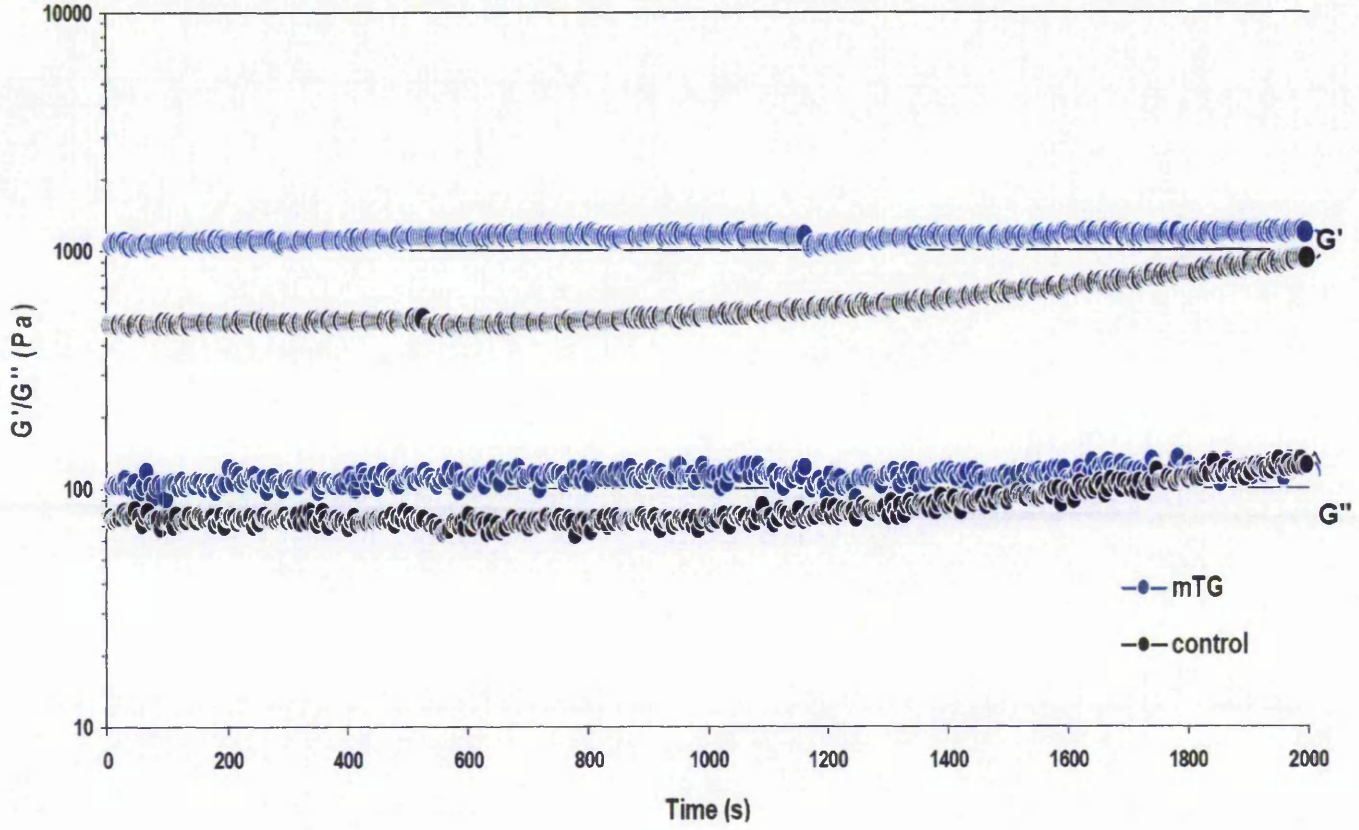

Figure 3.2.10. *In situ* fibrogenesis of native and mTG-treated collagen. The *in situ* fibrillogenesis characteristics were determined using a Bohlin C-VOR KTB30 rheometer fitted with a cylindrical Couette geometry set-up and 2cm diameter smooth plates as described in the Methods. Enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg. The plate-gap dislocation distance was set at 0.8mm with sample conditions maintained at 37°C.

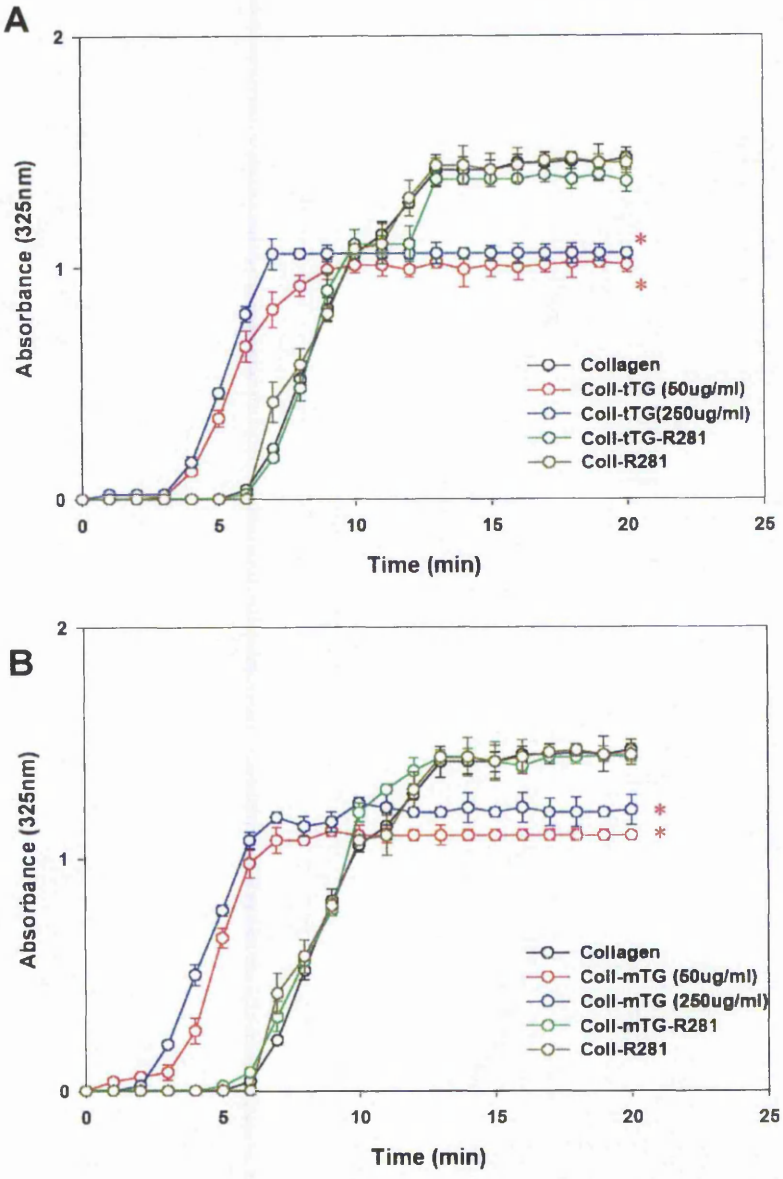

Figure 3.2.11. Fibrillogenesis of native and TG-treated collagen type I. The fibrillogenesis (via sample turbidity) of native collagen type I and collagen type I treated with tTG (**A**) and mTG (**B**) were monitored using silica cuvettes at 325nm wavelength (0.3mm slit width, 1.0nm bandwidth) in a PYE Unicam Recording SP1800 UV spectrophotometer with the temperature maintained at 25°C. Enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg. tTG and mTG were inactivated using the site-directed inhibitors, R283 and R281, respectively (500µM; 6 hours, 4°C). Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments. Results obtained following treatment with TG were compared to native collagen and values corresponding to $p<0.05$ when compared with the control are represented with a *. Line/data represented by a (red) coloured * indicate $p<0.05$ at all time points.

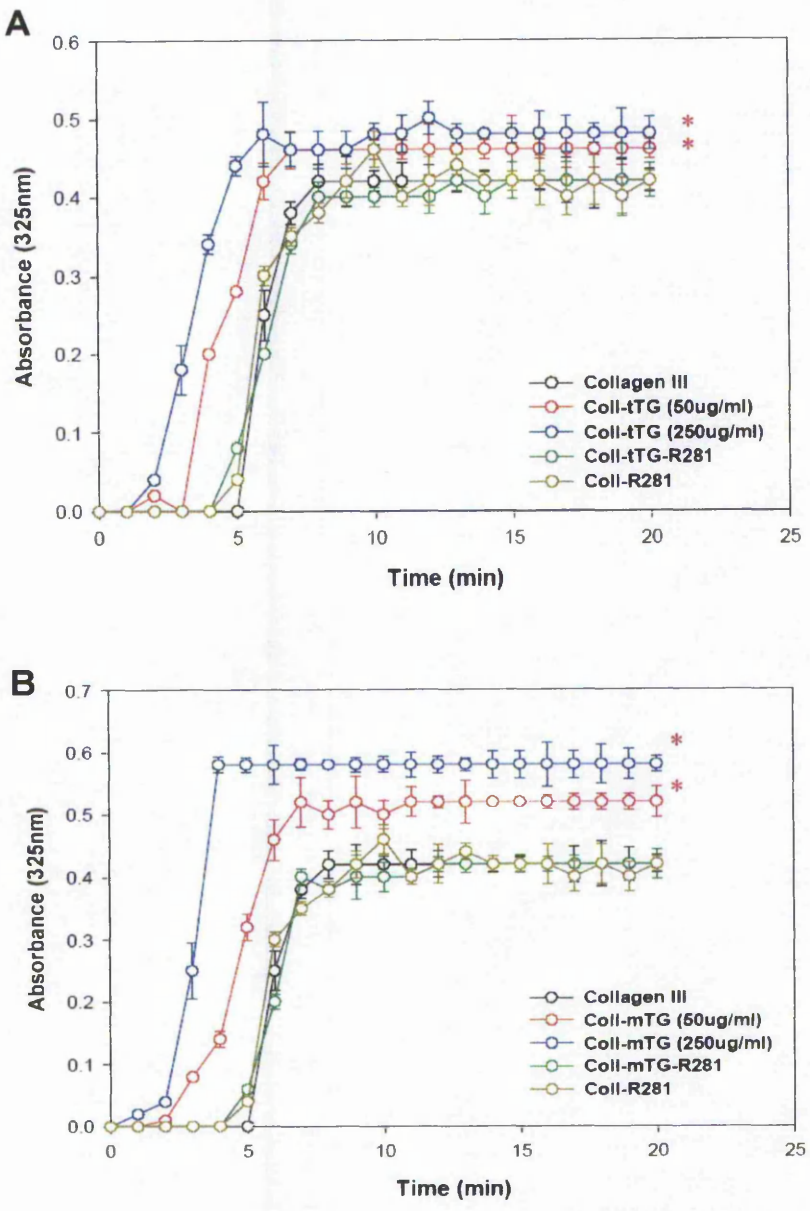

Figure 3.2.12. Fibrillogenesis of native and TG-treated collagen type III. The fibrillogenesis (via sample turbidity) of native collagen type III and collagen type III treated with tTG (**A**) and mTG (**B**) were monitored using silica cuvettes at 325nm wavelength (0.3mm slit width, 1.0nm bandwidth) in a PYE Unicam Recording SP1800 UV spectrophotometer with the temperature maintained at 25°C. Enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg. tTG and mTG were inactivated using the site-directed inhibitors, R283 and R281, respectively (500µM; 6 hours, 4°C). Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments. Results obtained following treatment with TG were compared to native collagen and values corresponding to $p<0.05$ when compared with the control are represented with a *. Line/data represented by a (red) coloured * indicate $p<0.05$ at all time points.

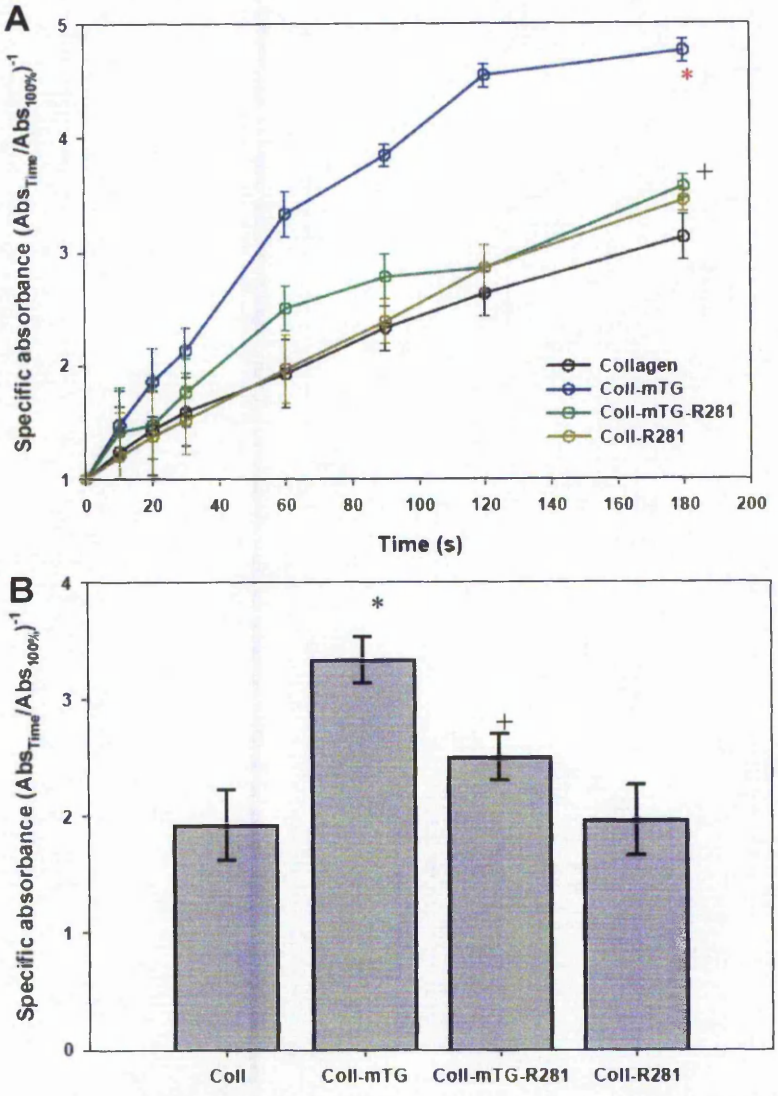

Figure 3.2.13. Antioxidant properties of native and mTG-treated collagen matrices. Collagen matrices were treated with 100µg/ml of mTG and subjected to the $ABTS^{\bullet+}$ free radical assay as described in the Methods. Enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg. The free-radical scavenging activity of the samples were monitored over a time course (**A**) or, at the 60s time point (**B**) and determined by decolourisation of $ABTS^{\bullet+}$ through the measurement of the reduction of the radical cation as percentage inhibition of the total absorbance at 734nm. mTG inactivation was achieved using the site-directed inhibitor, R281 (500µM for 6 hours at 4°C). Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments. The free-radical scavenging activity of TG-treated collagen was compared to native collagen and values corresponding to $p<0.05$ are represented with a *. Line/data represented by a (red) coloured * indicate $p<0.05$ at all time points. Results obtained in the presence of inhibitors were compared against the results in its absence and values corresponding to $p<0.05$ are represented with a +.

based solely on the TG activity. The same conclusion can be drawn in reflection of the assay at a specific time-point; that mTG-treated collagen is capable of absorbing significantly more free radicals than its control sample ($p<0.05$) as shown by Figure 3.2.13B.

3.2.8. Resistance of native and TG-treated collagen

The susceptibility of native and TG-treated collagen matrices to several collagenases was monitored by means of a fluorometric degradation assay. Figure 3.2.14A shows the susceptibility of collagen to microbial collagenase (*Clostridiopeptidase A*) before and after treatment with 500µg/ml of tTG and mTG. It can be seen that no difference exists between the native collagen and the tTG-treated collagen- both were degraded as expected. Interestingly, however, the collagen treated with 500µg/ml of mTG exhibits a significant resistance to degradation by the microbial collagenase ($p<0.05$) at the majority of time-points. Figure 3.2.14B demonstrates the susceptibility of the same matrices to HFDF (fibroblast; 48h culture on TCP) cell culture supernatant. It can be observed that a significant increase in the fluorescence occurs for the mTG-treated collagen ($p<0.05$), suggesting an increased susceptibility to degradation mediated by the HFDF culture supernatant is taking place. In comparison, collagen samples treated with tTG show only a slight increase in fluorescence detection ($p<0.05$) indicating that they are also degraded faster than the native form of collagen.

Figure 3.2.15 A and B shows the susceptibility of native and TG-treated collagen matrices to activated and purified MMP-1 and MMP-8, respectively. It can be deduced from Figure 3.2.15A, that a major increase in the fluorescence reading of the mTG-treated collagen is detected; indicating that on treating collagen with mTG, an increase in the susceptibility to MMP-1 degradation is experienced. In comparison, a small but significant ($p<0.05$) difference is noted between the native and the tTG-treated collagen; this behaviour implies that on treating the collagen with tTG, a protective effect to MMP degradation is experienced. A similar finding can be observed when the samples are treated with MMP-8, as shown in Figure 3.2.15B. In this example, it is deduced that on treatment with mTG, an increase in the susceptibility to MMP-8 degradation is experienced. Similarly, a small decrease ($p<0.05$) can

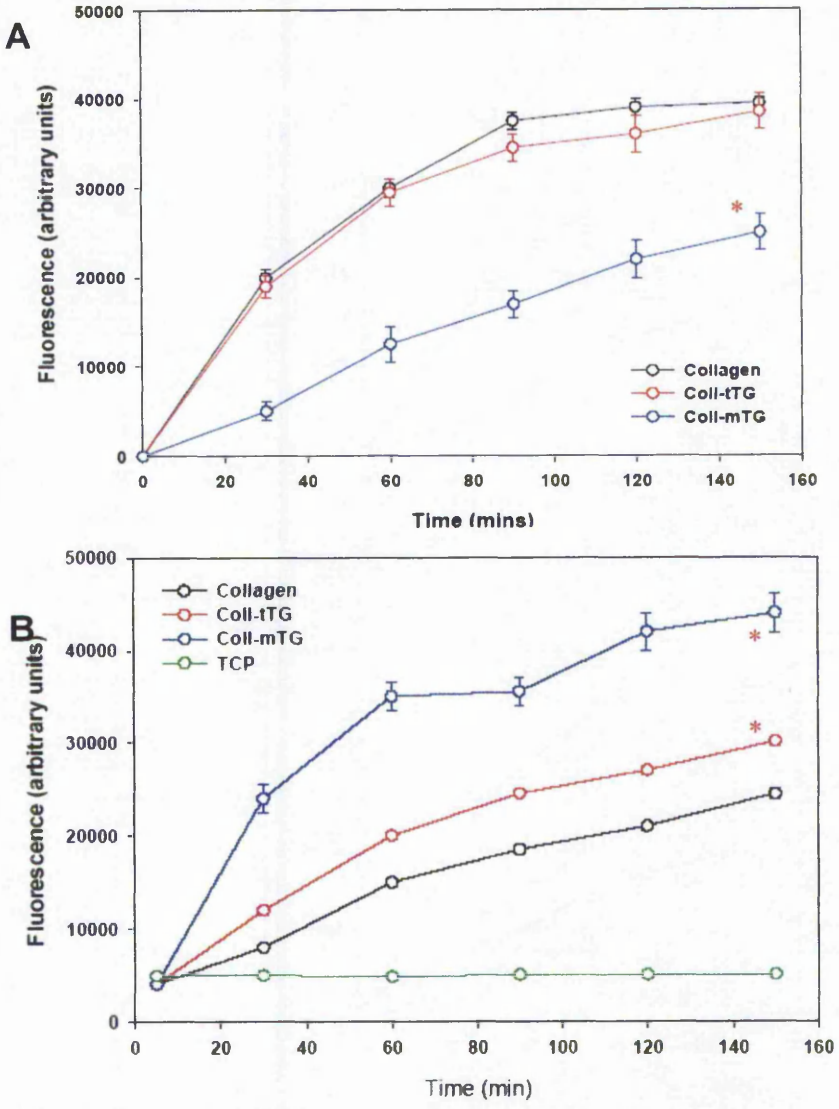

Figure 3.2.14. Susceptibility of native and TG-treated collagen to proteolytic degradation. FITC-labelled collagen (5mg/ml) was incubated overnight at 37°C, alone and with, either treatment with 0.5mg/ml of tTG or 0.5mg/ml mTG. Enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg. The collagen was then pelleted and washed as described in the Methods before being resuspended in 1ml of 50mM Tris-HCl pH 7.4, 5mM $CaCl_2$ containing: 20µg/ml *Clostridiopeptidase A* (**A**), cell culture supernatant from HFDF cells (48h culture period) used at a dilution of 1/20 (**B**). Digestion was performed at 37°C with constant shaking, from which 100µl samples were measured in a SpectraFluor® plate reader using an excitation and emission wavelength of 492nm and 535nm, respectively. Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments. The susceptibility to proteolytic degradation of the TG-treated collagens were compared to native collagen and values corresponding to $p<0.05$ are represented with a *. Line/data represented by a (red) coloured * indicate $p<0.05$ at all time points.

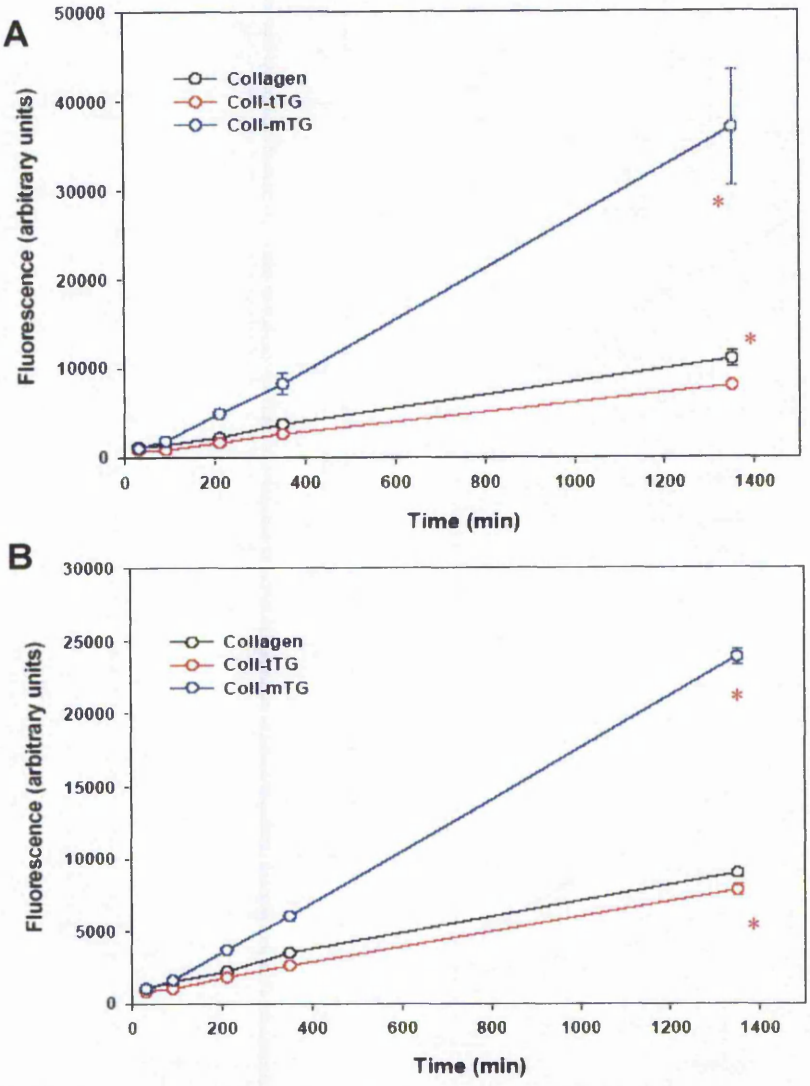

Figure 3.2.15. Susceptibility of native and TG-treated collagen to MMP degradation. FITC-labelled collagen (5mg/ml) was incubated overnight at 37°C, alone and with, either treatment with 0.5mg/ml of tTG or 0.5mg/ml mTG. Enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg. The collagen was then pelleted and washed as described in the Methods before being resuspended in 1ml of 50mM Tris-HCl pH 7.4, 5mM $CaCl_2$ containing: 50ng/ml of purified MMP-1 (**A**) or MMP-8 (**B**) in 50mM Tris-HCl (pH 7.4), 5mM $CaCl_2$, 0.005% (v/v) Brij-35, 1µM $ZnCl_2$. Digestion was performed at 37°C with constant shaking, from which 100µl samples were measured in a SpectraFluor® plate reader using an excitation and emission wavelength of 492nm and 535nm, respectively. Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments. The susceptibility to proteolytic degradation of the TG-treated collagens were compared to native collagen and values corresponding to $p<0.05$ are represented with a *. Line/data represented by a (red) coloured * indicate $p<0.05$ at all time points.

be observed for the tTG-treated collagen, suggesting a decrease in the susceptibility to degradation by MMP-8.

3.2.9. Collagen and gelatin zymography

The MMP profiles secreted by HFDF cells cultured on native and TG-treated collagen matrices were documented by collagen and gelatin zymography as shown in Figure 3.2.16 A and B, respectively. Following growth on native, untreated, type I collagen, HFDF cells show an induction of a wide array of collagenases and gelatinases when compared to growth on tissue culture plastic (TCP) alone. After growth on both tTG and mTG-cross-linked collagens, the induction of active MMP-1 (45kDa) is not as pronounced as compared to growth on native collagen; whereas the induction of active MMP-2 (66kDa) and MMP-9 (86kDa) were greatly increased in both cases-particularly when the cells were cultured on mTG-cross-linked collagen.

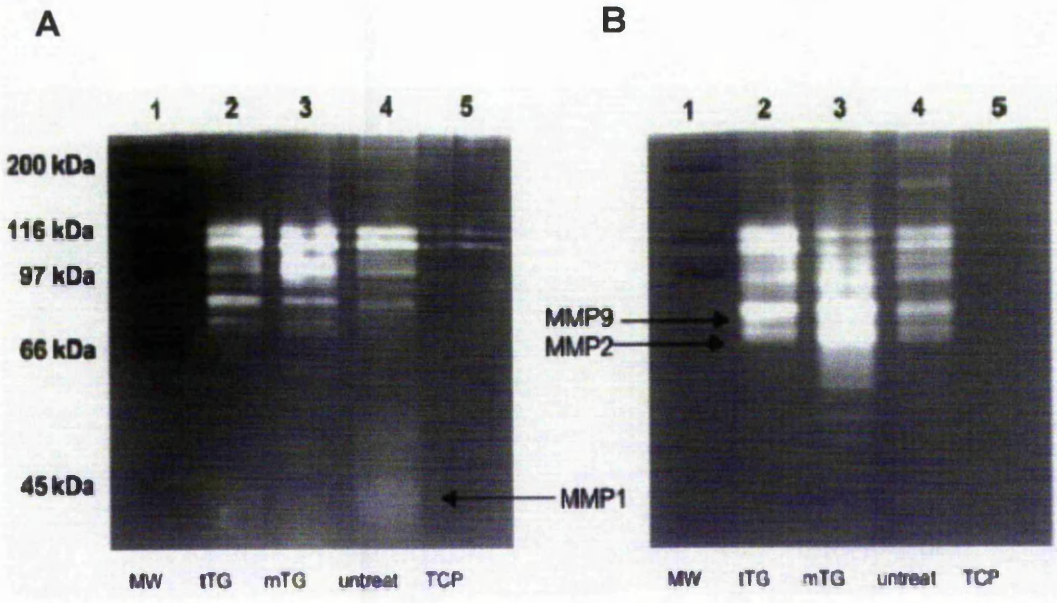

Figure 3.2.16. MMPs secreted by HFDF cells grown on native and TG-treated collagen matrices. Collagen (**A**) and gelatin (**B**) zymography of HFDF cell culture supernatants following 24h culture on the different substrates: native and 100µg/ml TG-treated collagen matrices. Enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg. Samples containing MMPs were diluted 1:1 with loading buffer (1M Tris-HCl, pH 6.8, 50% glycerol, 0.4% bromophenol blue) and fractionated at 100V in standard Laemmli running buffer (24mM Tris-HCl, 192mM glycine, 3.47mM SDS, pH 8.3), and on ice in a chilled-cabinet (4°C). Following electrophoresis, the gels were washed twice, with shaking, for 30 minutes each time in 200ml of wash buffer consisting of 2.5% (v/v) Triton X-100, to remove the SDS and recover MMP activity. The gels were then placed in digestion buffer (100mM Tris-HCl, 5mM $CaCl_2$, 0.005% Brij-35, 1µM $ZnCl_2$, 0.001% NaN_3, pH 8.0) for 16-48 hours at 37°C. Gels were then stained with 0.2% Coomassie brilliant blue R-250 in 50% ethanol, 10% acetic acid for 2h and de-stained by microwaving for 15min (full power; 850W) in 3 changes of distilled water. Lane 1, molecular weight markers (Sigma, Poole, UK); lane 2, tTG-treated collagen matrix; lane 3, mTG-treated collagen matrix; lane 4, untreated (native) collagen; lane 5, tissue culture plastic (no collagen).

3.3. DISCUSSION

It can be seen that distinct variations exist between the specific activities of TGs isolated from different organisms- in this case, between guinea pig liver tTG (gpl-tTG) and mTG isolated from *Streptomyces mobaraense*. The findings reported during this investigation appear to be consistent with observations noted by Shimba and co-workers who reported that, although the deamidation activity of mTG is weaker than that of fTG (analogous to tTG), the reaction rate and substrate specificity for the acyl donor of mTG are much higher than those of gpl-tTG- although no quantitative values were given in that report [Shimba *et al.*, 2002]. Differences in the variation of specific activity between the commercial and 'in-house' purified tTG can be explained by their relative storage and manufacture techniques: the 'in-house' enzyme had been isolated, purified and processed as fresh and as quickly as possible thus limiting the decrease in activity prior to use. On contrast, the commercial tTG has an unknown history; although it may have had the same initial corresponding activity for typical tTG, the long-term storage, processing and even delivery of the sample may have greatly affected its overall activity. The same reasoning can, thus, also be used to explain the presence of impurities, contaminants and degradation products seen in the SDS-PAGE and western blots for tTG. Likewise, in the case of mTG, the patented (commercial) preservation procedure may contribute towards the presence of degradation products or impurities.

The inhibition of tTG and mTG was achieved using the active-site specific irreversible inhibitors, R283 and R281. Inhibitor R283 is specifically designed to target FXIII (plasma transglutaminase) but affects other mammalian transglutaminases such as tTG. It is also is capable of passing through the cell membrane. The R281 inhibitor, unlike R283, was found capable of inhibiting both the mTG and tTG enzyme but cannot cross the cell membrane. Previous work within this laboratory has established IC50 values of ~30μM for both inhibitors for the tTG and a 95% inactivation of TG activity at concentrations of 250μM [Balklava, 2002; Jones *et al.*, 1997]. However, during this investigation, it was found that to achieve the 95% inactivation of tTG activity, 500μM treatment with the inhibitors was required. This concentration was therefore used throughout the investigation as a control for enzymatic activity. This loss of inhibition activity may be contributed to the long-term storage of the compounds or the type of assay used in their measurement as it, itself, a site-

directed inhibitor. Furthermore, it has also been suggested that the increased concentration requirement of the inhibitors may be as a direct consequence of the purity of the enzyme used: earlier work within this laboratory has utilised the commercial-sourced gpl-tTG. However, it has already been demonstrated that variations exist between the commercial and 'in-house' enzymes and, due to the higher purity of the source enzyme (indirectly implicating a higher specific activity), a higher concentration of inhibitor may be required to achieve the same degree of inhibition as previously observed.

The presence of small amounts of ε-(γ-glutamyl)lysine isopeptide bonds in native bovine collagen has been verified by ion-exchange chromatography analysis. On treating the collagen with transglutaminases, notable increases in the amount of isopeptide bonds were detected. In short, mTG is able to form more isopeptide bonds than tTG, per mg of protein, represented by the higher values of the mol cross-link to the mol of collagen ratio. As both variants of the enzyme are able to catalyse such a reaction, the difference in the reaction activity may be contributed by the physical characteristics of the mTG- which is almost half the size of the mammalian sourced enzyme. It may be that due to its smaller size, mTG is able to penetrate the collagen deeper and with more ease- thus able to crosslink a greater amount as opposed to the tTG, which is a significantly larger molecule- most likely catalysing bonds at the surface locality. Furthermore, it also feasible that a greater (molar) amount of mTG was utilised during the catalytic reaction due the variation in its molecular weight- approximately half the size of the mammalian counterpart. In short, a value of 2nmol of crosslink/mg of collagen protein, from 50µg/ml mTG treatment, appears to be consistent with previous work performed by this laboratory in other biotechnologically related mTG applications [Collighan *et al.*, 2002; Cortez *et al.*, 2002].

The self assembly of collagen type I (in solution) has been extensively studied both *in vitro* [Brightman *et al.*, 2000; Veis and George, 1994] and *in situ* [Birk *et al.*, 1988], although difficulties arose due to the gelation-transition mechanism involved. A similar behaviour can be observed in Figures 3.2.9 A and B which follows the dynamic visco-elastic behaviour of the native and mTG-treated collagen when subjected to varying frequency oscillations *in situ* as shown in Figure 3.2.10. In short, if a material expresses a G'>G" value, then the substance is more "solid-like". Contrastingly, if G">G', then it is

regarded as more like a "liquid". It can be deduced that on treating collagen with mTG, the gelation point of this new modified collagen is reached quicker- depicted by a more linear response and indicating a more gel-like characteristic. Since the initial collagen is already in the sol-state, applying more stress to the sample would cause a self-alignment response (organised arrangement) of the molecules. On monitoring the *in situ* fibrillogenesis behaviour of the gels, when subjected to an external stress (1Hz), it can be seen that gelation occurs almost immediately when mTG is added to the native collagen solution. On combining these observations, the results infer that on treating native collagen with mTG, a quicker gelation procedure occurs and ultimately leads to a stronger final gel matrix. This is further enforced by the fact that a bigger difference can be identified between G' and G" moduli. It is without a doubt that a change in the physical structure of the collagen occurs, which in turn, leads to changes of the characteristics and behaviour of the sample as clearly identified in the AFM images as shown in Figure 3.2.8.

It can be seen that for both of the TG-modified collagens, their fibril 'growth phase' are initiated at a much earlier time-point than in the native collagen and this behaviour also follows a dose-dependent response with respect to enzyme concentration. For both collagen type I and type III, it is observed that mTG provides the optimal change (i.e. time taken in the initiation of the growth phase) of the *in vitro* fibril assembly mechanism. Interestingly, an observation made by several investigators during the commencement of the fibril initiation period noted that, although a collagenous solution did not display any immediate turbidity reading (indicating that the large fibril structures have not yet been formed), unbanded microfibrils of approximately 3-5nm diameter were observed during the lag phase of the fibril assembly [Gelman *et al,* 1979]. Electron micrographs provided by Na and co-workers [Na *et al.*, 1986] during this stage of the fibrillogenesis process also bear a similar resemblance to the structures obtained during our investigation. Therefore, it may be deduced that on treatment with TGs, the earlier onset of fibril assembly may be as a direct consequence of the premature formation of microfibrils and this may be a contributing factor to the changes in the observed turbidity results.

Another interesting observation can be noted in this study regarding the final level of fibril formation reached by the samples: in the case of the type I collagen, a decrease (lower level of turbidity) in the final level of fibril formation

was noted with both the TG-treatments when compared to that of the native collagen. In contrast, for the collagen type III samples, an increase in the final level of fibril formation was noted. On surveying the literature, it is surprising to find that a similar observation had been observed earlier by McPherson and his colleagues who reported that the solution turbidity of fibrils assembled from (pepsin-solubilised) bovine collagen was inversely related to the rate of fibril formation [McPherson *et al.,* 1985]. In that morphological study, the investigators concluded that lower solution turbidity could be attributed to a reduction in fibril diameter and further postulated that the collagen may either have generally formed shorter fibrils, or were more densely packed and, hence, were able to scatter less light [McPherson *et al.,* 1985]. This hypothesis could explain the observation noted in the AFM results whereby mTG-treated fibrils are much narrower and closely packed than those found on the native collagen. This hypothesis may also explain the relative changes to the terminal turbidity level of the samples.

The discrepancy noted between the final turbidity level of the type I and III collagen may be attributed purely to their physical characteristics; type I collagen is a heterotrimer and normally consists of two α1(I) chains and one α2(I) chain, whereas type III collagen, a homotrimer, consists of three identical α1(III) chains [Vuorio and de Crombrugghe, 1990]. Not only has it been found that the collagen type I molecules are relatively shorter than the type III subunits [Cameron *et al.,* 2002] but differences between the fibril diameter of the two types of collagen also exist [Jussila *et al.,* 2004]. As such, type III collagen appears to be more 'swollen' than a similar type I fibril and the resulting (relative) size difference may correspond to the degree of turbidity exerted by the sample. The *in vitro* fibril formation of native collagen type I and III has already been investigated by several researchers and the results are comparable to the findings outlined here [Taatjes *et al.*, 1999; Nemeth-Csoka and Kovacsay, 1979].

On treating collagen type I with TGs, we have demonstrated an increase in the free radical scavenging (antioxidant) characteristics of the modified collagen as compared to the native collagen occurs. Hence, it is possible that the increased ability to 'absorb' more free radicals is solely based on the modified physical characteristics of the TG-treated collagen, either as a direct consequence of the ε-(γ-glutamyl)lysine cross-links reinforcing the 'strength' of

the material and preventing concomitant fragmentation or, alternatively, the formation of a denser and highly cross-linked 'porous' structure which may be capable of entrapping the free radicals- as implied and inferred upon from the AFM images, or indeed as a combination of both aspects. Recently, it has been demonstrated that native collagen type I was capable of inhibiting $^{-}OH^{\bullet}$-mediated apoptosis by scavenging free radicals and, in the same study, the investigators also showed that denatured collagen and gelatin lacked the same antioxidative and antiapoptotic effects [He *et al.,* 2002]. Hence, it may be postulated that such characteristics may well be attributed to the physical aspects of the collagen cross-linked network.

Although it has been well documented that on increasing the amount of cross-links in native collagen, an enhancement in the resistance to cell secreted proteases is observed [Geiger and Friess, 2002]; a contrasting situation can be identified with the results presented here which propose that the modified collagen substrates may be *species- specific*, that is to say, collagen modified by mTG is less susceptible to degradation by microbial-sourced enzymes (i.e. *Clostridiopeptidase A)* and, that collagen modified by tTG is less susceptible to degradation by the mammalian MMPs and cell culture supernatant. This phenomenon has yet to be documented in the current literature but a provisional explanation may be considered: it has been previously reported that the mammalian collagenases; MMP-1, MMP-3 and MMP-8 can attach to collagen at unique cleavage sites (a Gly-Leu or a Gly-Ile bond followed by either an Ala or a Leu residue) and that analyses of these cleavage sites from different collagen sequences reveal that they all share similar features [McDonnel *et al.*, 1995; Fields, 1991]. Hence, it could be postulated that the imino-poor regions near the collagenase cleavage sites become modified and affect the flexibility of the collagen fibril in a certain manner as to change its specificity to collagenase recognition [Fields, 1991]. Furthermore, as it has now been demonstrated that TGs treatment is able to cross-link native collagen by the formation of stable isopeptide linkages- a reduction in the degradation mechanism may occur due to the direct/indirect consequence of the cross-link masking the (previously) exposed active-site. Moreover, this could also be linked to the possible different cross-linking sites of the two enzymes [Motoki *et al.*, 1992].

Additionally, the functional, morphological and metabolic characteristics of fibroblasts are very dependent on their culture environment; previous studies have shown that fibroblasts cultured in 3-D collagen matrices are very distinct from those cultured as monolayers; in fact, changes in gel mechanics can lead to differential expression of wound associated genes [Phillips *et al.*, 2003; Girton *et al.*, 2002; Kessler *et al.*, 2001; Huang and Ingber, 1999]. This observation would provide another possible hypothesis for the results seen here: that the TG-modified collagens have experienced sufficient physical changes, causing a change in the surrounding culture 'environment' for the cells and, hence, may lead to induce the expression of different genes and/or other proteolytic enzymes and proteins. This hypothesis is further strengthened by the observations noted in the zymographs, as seen in Figure 3.2.14, whereby up-regulation of MMP-2 and MMP-9 and the down-regulation of MMP-1 are experienced when HFDF cells are cultured on the TG-treated collagen matrices.

CHAPTER 4:
BIOLOGICAL CHARACTERISATION

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4.1. INTRODUCTION

In contrast to the findings by Jelenska and colleagues who reported that cross-linking did not occur when native collagen was treated with gpl-tTG [Jelenska *et al.*, 1980]; we demonstrated, in the previous chapter, that such modifications, via ε-(γ-glutamyl)lysine isopeptide bonding, do occur following the treatment of native collagen with both tTG and mTG in a dose-dependent manner. As a consequence of this modification, the collagen not only expressed enhanced mechanical properties but also allowed the onset of fibrillogenesis to occur at a much earlier time point. Furthermore, the evidence also demonstrated that the modified-collagen expressed a greater resistance to proteolytic attack in a species-specific manner. However, the effect of the TG-modification on the collagen in terms of the biological function, biocompatibility and the associated cellular response has not been well elucidated- as it has been previously reported that changes to the structure of collagen may possibly distort or disrupt the interaction with cells during its use as a biomaterial [Goo *et al.*, 2003; van Wachem *et al.*, 1996; Weadock *et al.,* 1995; Kato and Silver, 1990; Huang-Lee *et al.*, 1990; Cavallaro *et al.,* 1984].

The majority of interactions between cells and an implant material occur on the surface of the aforementioned biomaterial and, hence, much attention has been focused on defining the characteristics that may promote such desirable reactions [Griffith and Naughton, 2002]. The adhesion of cells to the ECM (or material surface) is a basic cellular process but is often seen as the most important and fundamental factor- capable of influencing and regulating cell growth, survival, migration and differentiation [Martin *et al.*, 2002; Schwartz and Ginsberg, 2002; Howe *et al.*, 1998; Gumbiner, 1996] as well as being involved in processes such as morphogenesis and wound healing [Anselme, 2000; Majeska *et al.,* 1993; Burridge and Fath, 1989]. This adhesion process is mediated by the interaction of a class of heterodimeric transmembrane cell receptors known as the integrins that bind selectively to different proteins of the ECM [Ruoslahti and Pierschbacher, 1987; Hynes, 1987 and 1992]. Furthermore, signal transduction by the ECM (substrate) has been shown to also be mediated through the integrin receptors [Salter *et al.*, 1997; Akiyama, 1996] and the exact nature of this interaction is extremely important for the majority of cellular activity. Therefore, the characteristics of an ideal

biomaterial surface should provide conditions that stimulate and promote desirable cell-ECM interactions [Verrier *et al.*, 2002].

Even though natural components (i.e. collagen) are regarded as suitable and sufficient when utilised as biomaterials, researchers are still currently devising new technologies to enhance their ability as possible tissue substitutes: with the most successful methodologies that involve the grafting of complete ECM proteins (e.g. fibronectin, vitronectin) [Kantlehner *et al.*, 2000; Hern and Hubbell, 1998] or, small peptides fragments (e.g. Arg-Gly-Asp; the RGD-binding motif) to the native biomaterial [Pakalns *et al.*, 1999; Mann *et al.*, 1999; Grzesiak *et al.*, 1997; Clark, 1985; Pierschbacher and Ruoslahti, 1984].

The aim of this chapter is to characterise the biological effects of the modified collagen with respect to cell spreading, attachment, proliferation, migration and differentiation for both HFDF and HOB cells (i.e. for soft and hard tissue applications respectively) and whether this biomaterial induces apoptosis or premature cell death. Furthermore, the possibility of grafting additional synthetic and/or natural ECM proteins to the modified-collagen, with a view of further enhancing its biocompatibility, will also be investigated.

4.2. RESULTS

4.2.1. Resistance of matrices to HOB cell mediated degradation

Degradation of native collagen I by HOB cells (visualised as lighter blue areas) occurred just 24 hours after the cells were cultured on the matrix as seen in Figure 4.2.1A. In contrast with matrices that were treated with tTG and mTG, degradation occurred at a much slower rate- with a higher amount of residual collagen remaining, as judged by the amount of Coomassie blue stained areas left in the field of view. Hence, collagen treated with 50µg/ml tTG or 50µg/ml mTG showed greater resistance to HOB-cell-mediated degradation as compared to the native collagen. Comparison of the residual blue staining suggests that the mTG-treated collagen demonstrates a slightly higher degree of resilience to HOB-cell-mediated degradation as opposed to the tTG-treated collagen. To confirm the data observed during the staining assay, the residual (collagen) protein concentration was assessed after its solubilisation by proteolytic digestion and shown in Figure 4.2.1B. This confirmed quantitatively and significantly ($p<0.05$) that an increase in the resistances, to HOB-cell-mediated degradation, of the TG-cross-linked collagens exists. However, very little difference was observed between the collagens cross-linked by the two different transglutaminases in this respect.

The degradation of native collagen by HFDF cells was also assessed in the same manner and found to occur after 24 hours of cell growth. Almost negligible collagen remaining after 72 hours as pictured in Figure 4.2.2A. In contrast, in the collagen matrices that were treated with tTG or mTG, degradation occurred at a much slower rate and resulted in a much higher amount of residual collagen as detailed by the Coomassie blue staining. As such, collagen treated with 50µg/ml tTG or 50µg/ml mTG showed greater resistance to HFDF-cell-mediated degradation as compared to the native collagen. As with the HOB cells, the residual protein concentration was assessed post-proteolytic degradation and indicated that there is a decrease in the susceptibility to cell mediated degradation following TG-treatment (Figure 4.2.2B). However, for the HFDF cells, a significant difference ($p<0.05$) can be identified between the collagen cross-linked by the different transglutaminases- with the mTG-treated collagen showing greater resistance to HFDF-cell-mediated degradation than the tTG-treated variant. Both sets of results can be seen summarised in Table 4.2.1.

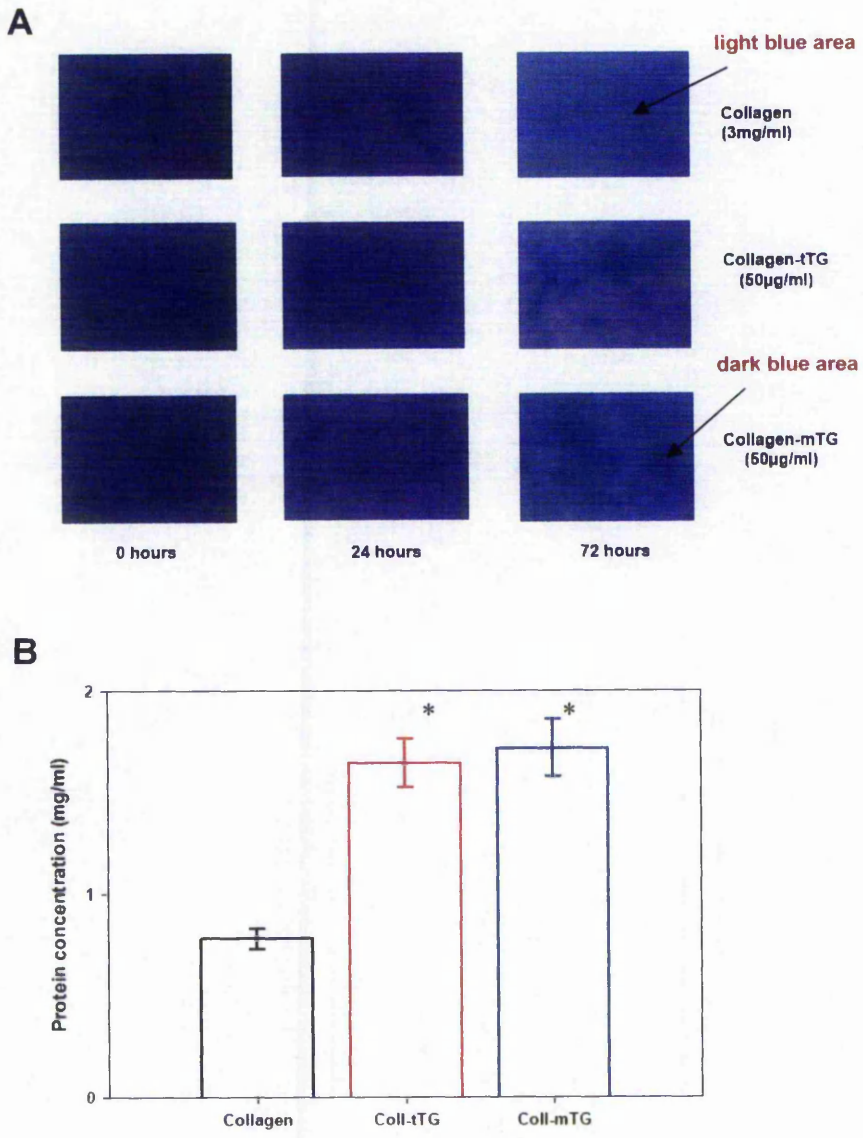


Figure 4.2.1. Degradation of native and TG-treated collagen I by HOB cells. Images of the residual collagen remained in native and TG-treated matrices, stained with Coomassie blue, following 72h culture with (**A**) 2 x 10^4 HOB cells. Enzyme activities: tTG: 17200 Units/mg; mTG: 18700 Units/mg. Cells were removed with sodium deoxycholate and the matrices treated to proteolytic action with microbial collagenase and trypsin. (**B**) Residual solubilised protein concentration of matrices following 72h of HOB cell culture measured using the Lowry assay as described in the Methods section. Initial collagen levels were 3mg/ml. Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments each with triplicate samples. Results obtained following treatment with TG were compared to native collagen and values corresponding to $p<0.05$ when compared with the control are represented with a *.

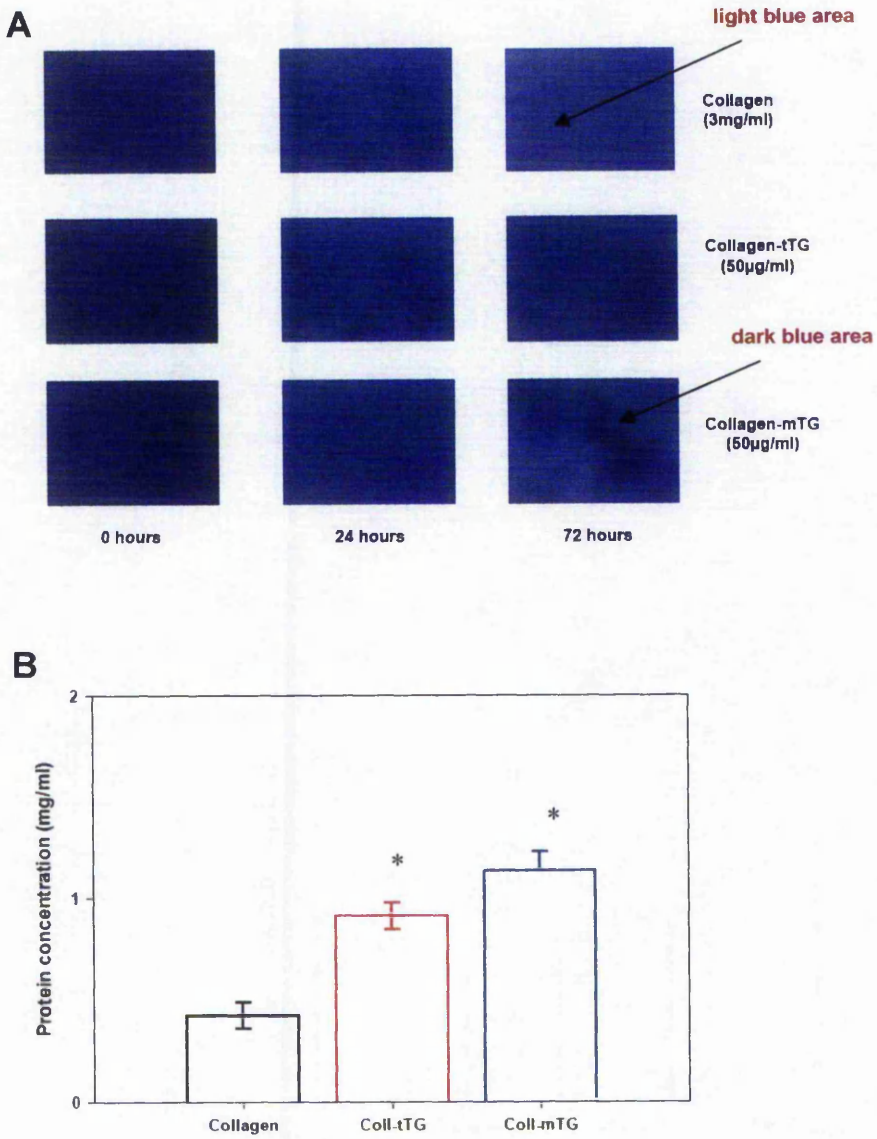


Figure 4.2.2. Degradation of native and TG-treated collagen I by HFDF cells. Images of the residual collagen in native and TG-treated matrices, stained with Coomassie blue, following 72h culture with (**A**) 2 x 10^4 HFDF cells. Enzyme activities: tTG: 17200 Units/mg; mTG: 18700 Units/mg. Cells were removed with sodium deoxycholate and the matrices treated to proteolytic action with microbial collagenase and trypsin. (**B**) Residual solubilised protein concentration of matrices following 72h of HFDF cell culture measured using the Lowry assay as described in the Methods section. Initial collagen levels were 3mg/ml. Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments each with triplicate samples. Results obtained following treatment with TG were compared to native collagen and values corresponding to $p<0.05$ when compared with the control are represented with a *.

Cell line	Collagen	Coll-tTG (50µg/ml)	Coll-mTG (50µg/ml)
HOB	24% ±3.1	55% ±1.9 *	59% ±2.1 *
HFDF	14% ±2.6	30% ±2.3 *	38% ±2.5 *

Table 4.2.1. Degradation of native and TG-treated collagen I by HOB and HFDF cells. Following 72h culture on the appropriate substrate, cells were removed with sodium deoxycholate and matrices treated by proteolytic action with microbial collagenase and trypsin. The residual solubilised protein was measured and expressed as a percentage of the initial level. Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments each with triplicate samples. Results obtained following treatment with TG were compared to native collagen and values corresponding to $p<0.05$ when compared with the control are represented with a *.

4.2.2. Inhibition of cell mediated degradation

To assess the extent of the inhibition of cell-mediated degradation, the fluorescence of native and TG-treated (50µg/ml) FITC-collagen I following HFDF-cell-mediated degradation, was monitored. Additionally, further treatment in this experiment was via the addition of a broad range MMP inhibitor to the collagen with the corresponding negative control. It can be seen (Figure 4.2.3) that on inhibiting MMP activity, a higher amount of residual FITC-collagen is detected in both the native and TG-treated matrices, emphasising that the cell-mediated degradation experienced is in part a direct consequence of MMP activity. Furthermore, the tTG and mTG-treated matrices maintained significantly higher residual fluorescence readings ($p<0.05$) than the native FITC-collagen suggesting that these matrices possess a greater physical advantage by being more resistant to cell-mediated degradation. Samples treated with tTG demonstrated the highest resistance to HFDF-cell-mediated degradation.

4.2.3. Attachment and spreading characteristics of HOB and HFDF cells on native and TG-treated collagen substrates

Figures 4.2.4, 4.2.5 and 4.2.6 show the short-term (6-hour) cell-attachment characteristics of HOB and HFDF cells when cultured on native and TG-treated collagen I. It can be seen that increased numbers of both the HOB and the HFDF cells attached when cultured on transglutaminase cross-linked collagen when compared to that on the native collagen I substrate. For the HOB cells, a comparable cell attachment profile was observed on both the 50µg/ml and 100µg/ml TG-treated collagens (Figure 4.2.5A and B) giving a significant increase of around ~20% in the number of attached cells for the corresponding time points over the non-crosslinked collagen ($p<0.05$). Comparable enhancements in cell attachment on the 50µg/ml and 100µg/ml TG-cross-linked collagens were also observed for the HFDF cells ($p< 0.05$) as shown in Figures 4.2.6A and B. In addition, Figures 4.2.4, 4.2.7 and 4.2.8 show the short-term (1h and 6h time points) spreading characteristics of the HOB and the HFDF cells when cultured on native and TG-treated collagen substrates. An increase in the number of spread cells occurred when cultured on 50µg/ml transglutaminase cross-linked collagen. In the case of HOB cells,

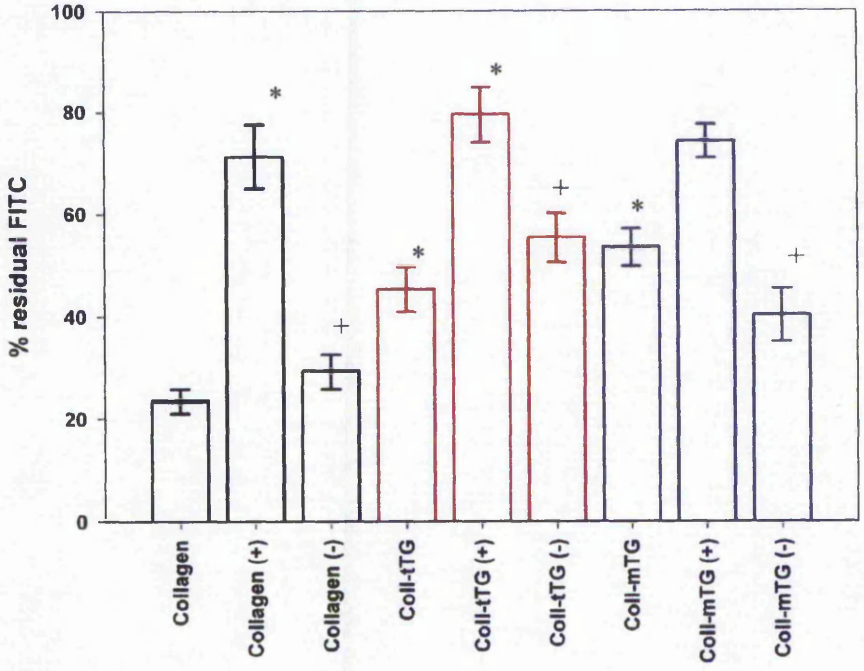


Figure 4.2.3. Inhibition of the degradation of native and TG-treated collagen I by HFDF cells. 5mg/ml of FITC-labelled collagen was incubated overnight at 37°C, alone and treatment with either 50µg/ml of tTG or 50µg/ml mTG. Enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg. 2ml of cell-suspension ($2x10^5$ cells/ml) containing 25µM GM6001 broad range inhibitor (Galardin; N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl0]-L-tryptophan methylamide) or 25µM of the negative control (N-t-butocarbonyl-L-leucyl-L-tryptophan methylamide) (CalBiochem, Nottingham, UK) was seeded on to the relevant FITC-labelled collagen substrate and maintained in a humidified-atmosphere incubator at 37°C and with 5% (v/v) CO_2. Following 2h of culture, 75µl of supernatant was removed and the fluorescence measured in a SpectraFluor® plate reader using an excitation and emission wavelength of 492nm and 535nm, respectively. Values are expressed as the % residual FITC in the collagen matrix based on the total fluorescence of the matrix (determined by degradation of control collagen matrices using microbial collagenase and trypsin). (+) represents samples treated with 25µM GM6001 broad range MMP inhibitor; (-) represents samples treated with 25µM of the negative control. Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments. The inhibition of degradation of the TG-treated collagen was compared to native collagen and values corresponding to $p<0.05$ are represented with a *. Results obtained in the presence of inhibitors were compared against the results in its absence and values corresponding to $p<0.05$ are represented with a +.

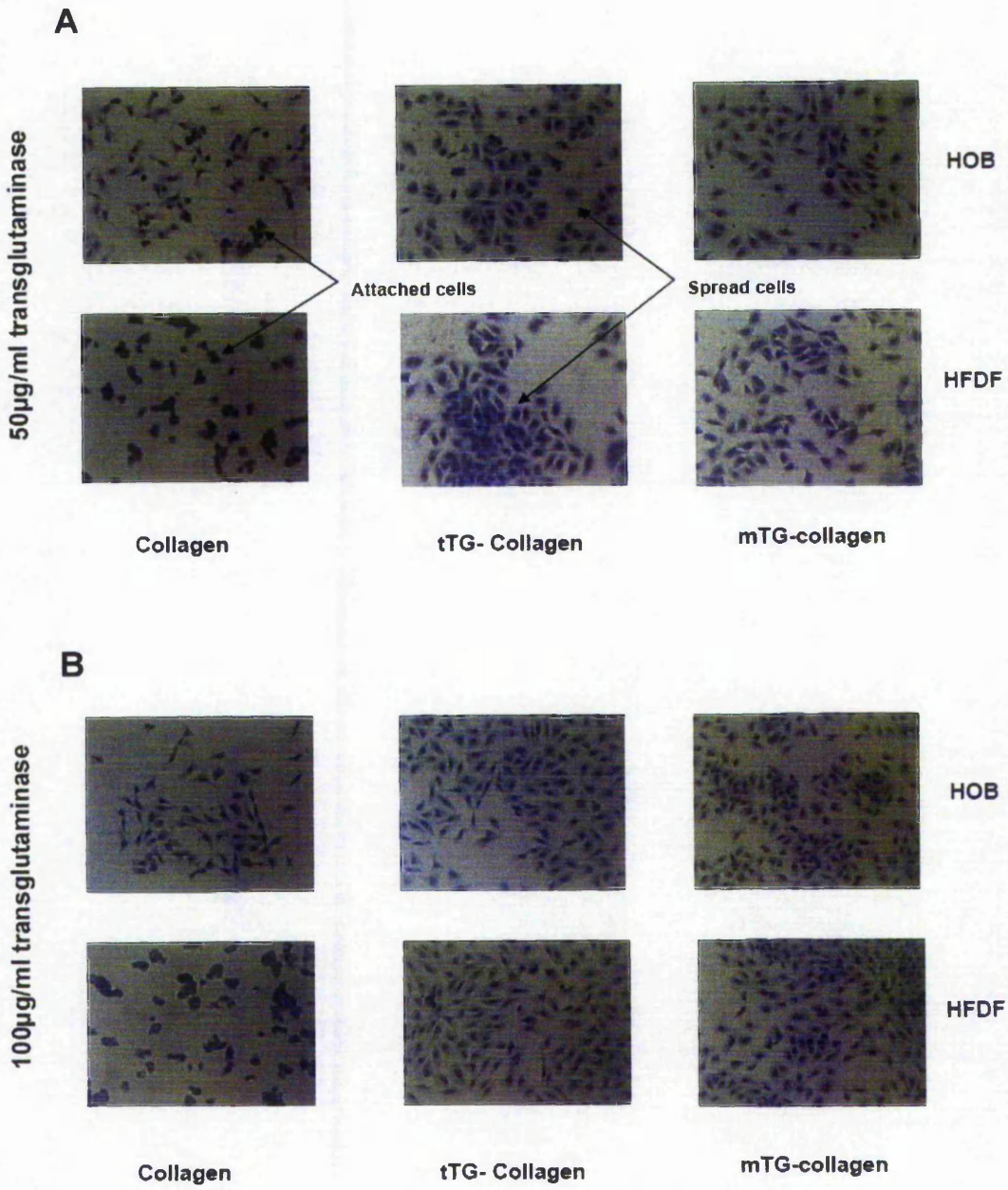


Figure 4.2.4. Attachment and spreading of HOB and HFDF cells on native and TG-treated collagen I substrates. Images of cells (2 x 10^5 cells seeded initially) following 6h incubation, on native and TG-treated collagen substrates. Cells were fixed using 3.7% (w/v) paraformaldehyde before being stained with May-Grunwald and Giemsa stains and then viewed at x400 magnification (**A**) 50µg/ml TG and (**B**) 100µg/ml TG. Enzyme activities: tTG: 17200 Units/mg; mTG: 18700 Units/mg.

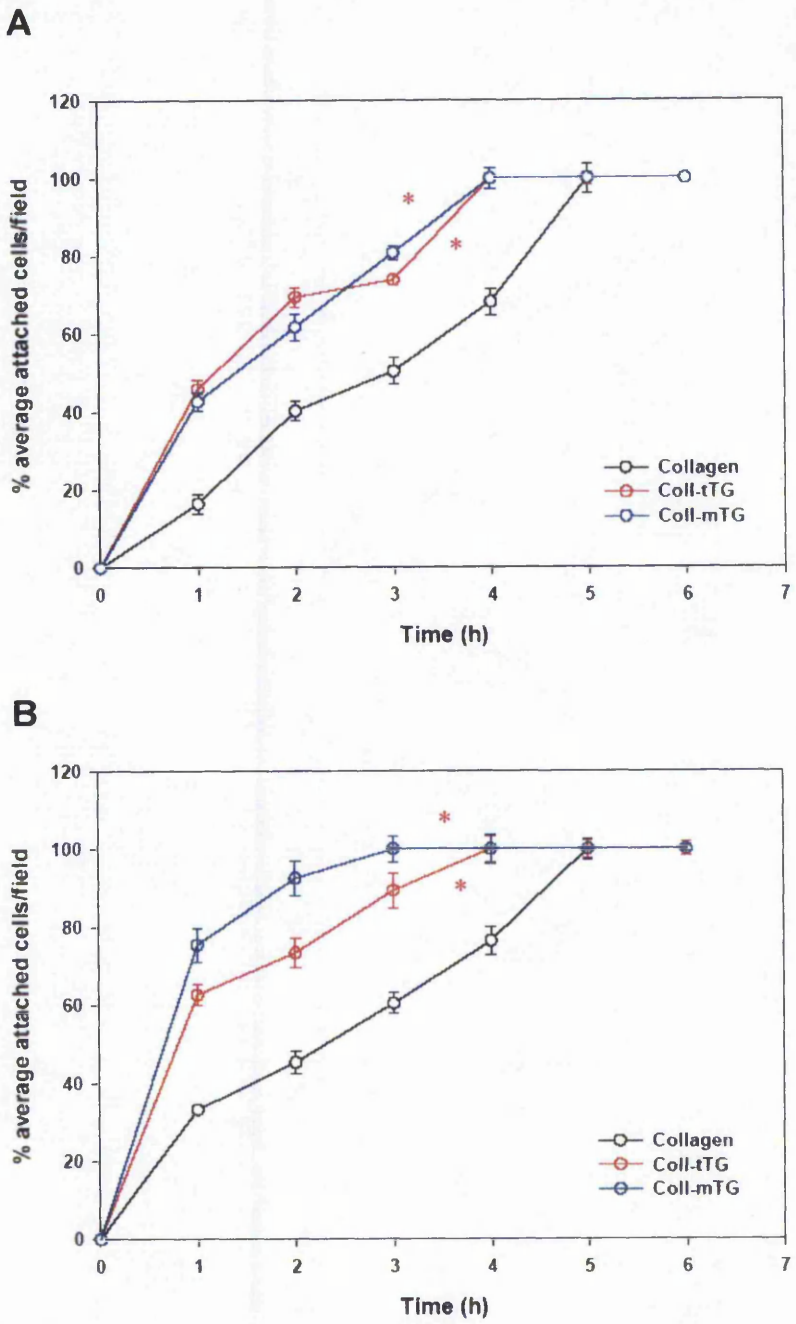


Figure 4.2.5. Attachment of HOB cells on native and TG-treated collagen I substrates. $2x10^4$ HOB cells cultured on native, (**A**) 50µg/ml TG and (**B**) 100µg/ml TG treated collagen matrices. Attachment assay was performed as described in the Methods. Enzyme activities: tTG: 17200 Units/mg; mTG: 18700 Units/mg. Attachment is expressed as a percentage of the total number of cells after 6h. Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from four independent experiments each with triplicate samples. Results obtained following treatment with TG were compared to native collagen and values corresponding to $p<0.05$ when compared with the control are represented with a *. Line/data represented by a (red) coloured * indicate $p<0.05$ at all time points.

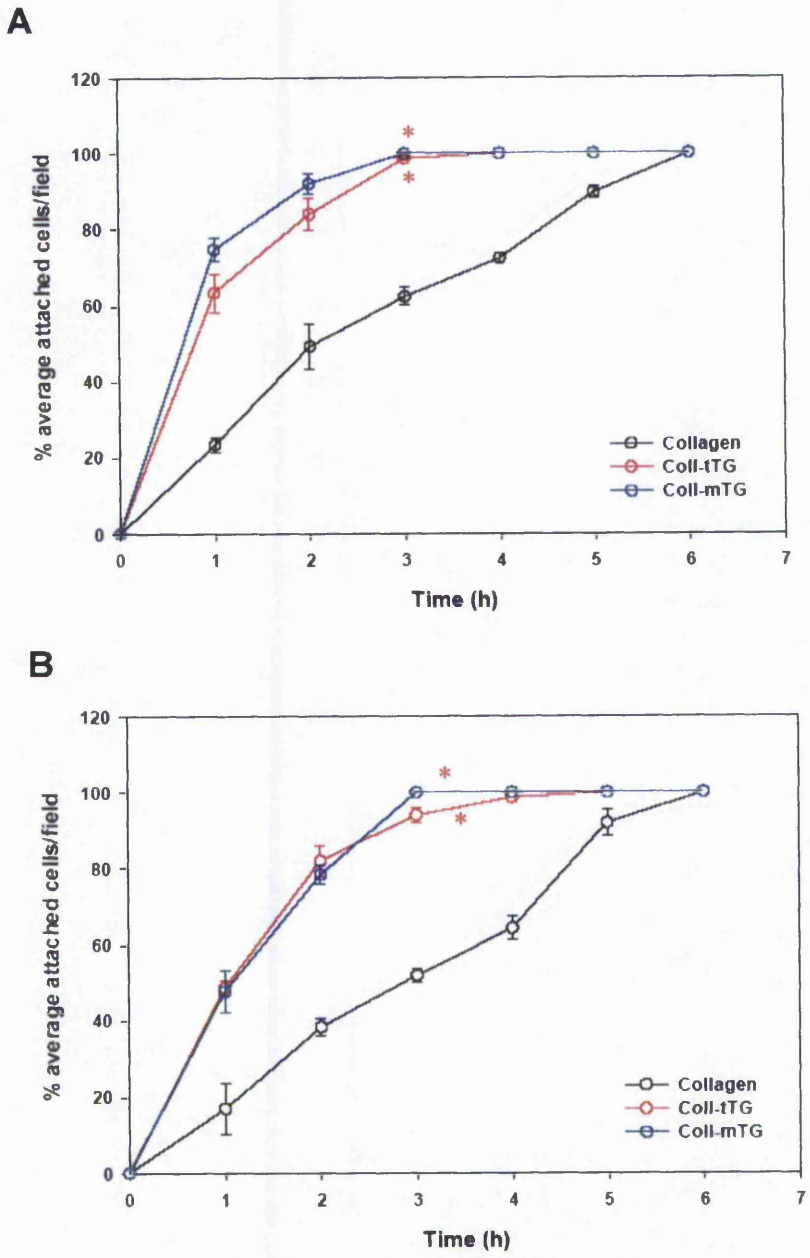


Figure 4.2.6. Attachment of HFDF cells on native and TG-treated collagen I substrates. $2x10^4$ HFDF cells cultured on native, (**A**) 50µg/ml TG and (**B**) 100µg/ml TG treated collagen matrices. Attachment assay was performed as described in the Methods. Enzyme activities: tTG: 17200 Units/mg; mTG: 18700 Units/mg. Attachment is expressed as a percentage of the total number of cells after 6h. Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from four independent experiments each with triplicate samples. Results obtained following treatment with TG were compared to native collagen and values corresponding to $p<0.05$ when compared with the control are represented with a *. Line/data represented by a (red) coloured * indicate $p<0.05$ at all time points.

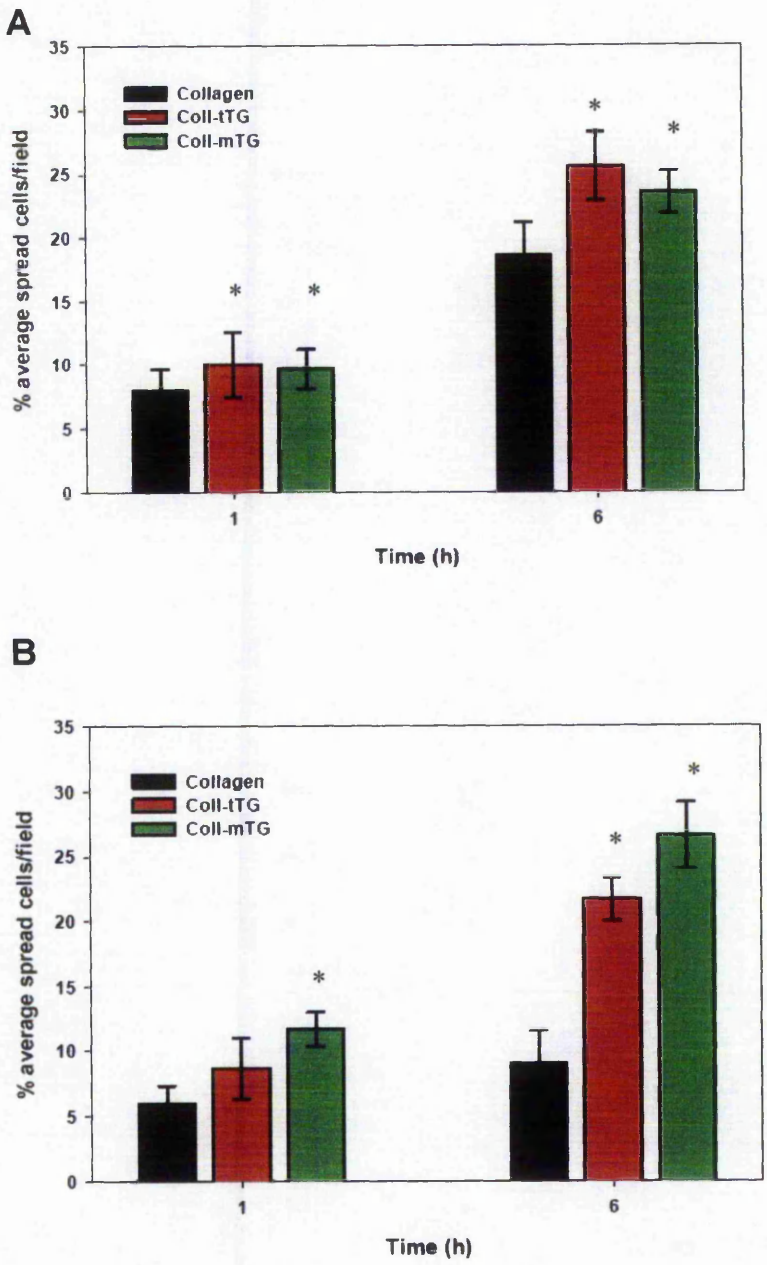


Figure 4.2.7. Spreading of HOB cells on native and TG-treated collagen I substrates. $2x10^4$ cells HOB were cultured for 1 and 6h on native, (**A**) 50µg/ml TG (**B**) 100µg/ml TG treated matrices as described in the Methods. Enzyme activities: tTG: 17200 Units/mg; mTG: 18700 Units/mg. Spreading is expressed as a percentage of the total number of cells in the field of view. Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from four independent experiments each with triplicate samples. Results obtained following treatment with TG were compared to native collagen and values corresponding to $p<0.05$ when compared with the control are represented with a *.

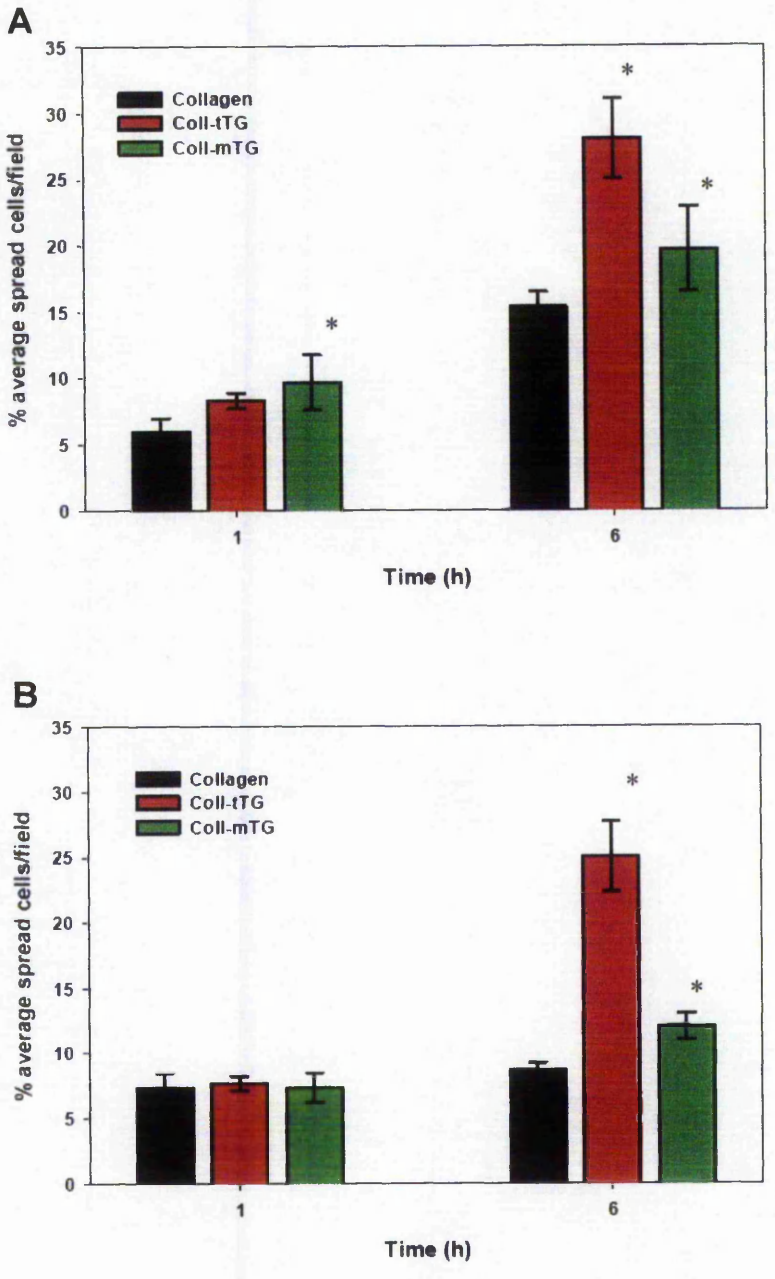


Figure 4.2.8. Spreading of HFDF cells on native and TG-treated collagen I substrates. $2x10^4$ cells HFDF were cultured for 1 and 6h on native, (**A**) 50µg/ml TG (**B**) 100µg/ml TG treated matrices as described in the Methods. Enzyme activities: tTG: 17200 Units/mg; mTG: 18700 Units/mg. Spreading is expressed as a percentage of the total number of cells in the field of view. Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from four independent experiments each with triplicate samples. Results obtained following treatment with TG were compared to native collagen and values corresponding to $p<0.05$ when compared with the control are represented with a *.

only a comparable increase of ~5% in the spreading of the HOB cells, at each time-point, is seen on the tTG and mTG-treated collagen substrates (Figure 4.2.7A). In contrast, the HFDF cells showed a significantly different cell spreading profile on the 50µg/ml TG-treated collagen- with increases of at least 10% ($p< 0.05$) observed for both of the TG-treated variants as documented by Figure 4.2.8A.

A potential dose-dependent response was noted for the number of spread cells observed on the 100µg/ml transglutaminase cross-linked collagen. In the case of HOB cells, an increase of approximately 10% can be observed in spread cells at the 1h time-point and this behaviour increased greatly with increasing time of culture (Figure 4.2.7B). In contrast, with the HFDF cells, no significant differences were noted for the spreading characteristics at the start of culture ($p<0.05$) between the native and TG-treated matrices but, as culture time increased, a much more distinct and significant behaviour became apparent on the tTG treated collagen; the spread cells increasing by ~25% at the 6h time-point (Figure 4.2.8B). In contrast, the microbial-TG treated collagen showed only a slight improvement in the spreading characteristics of cells ($p< 0.05$) compared to the native collagen substrate.

4.2.4. Proliferation characteristics of HOB and HFDF cells on native and TG-treated collagen substrates

The proliferation characteristics of the cells were monitored with the CellTiter Cell proliferation assay kit (Promega, UK) over a 196-hour culture period. It can be seen from Figures 4.2.9 and 4.2.10 that the proliferation rates of both the HOB and the HFDF cell lines were enhanced on the TG-treated collagen substrates when compared to that of with the native collagen. In the case of the HOB cells, no significant difference ($p<0.05$) in the proliferation characteristics were observed with either of the TG-treated collagen substrates at 50µg/ml or 100µg/ml concentrations (Figure 4.2.9A and B). In contrast, the HFDF cells showed an extended and significant period of enhanced proliferation ($p<0.05$) when cultured on collagen treated with 50µg/ml TG (up to 24h) as well as 100µg/ml TG (up to 48h) as seen in Figures 4.2.10A and B, respectively. This trend was maintained further for approximately 168 hours of culture.

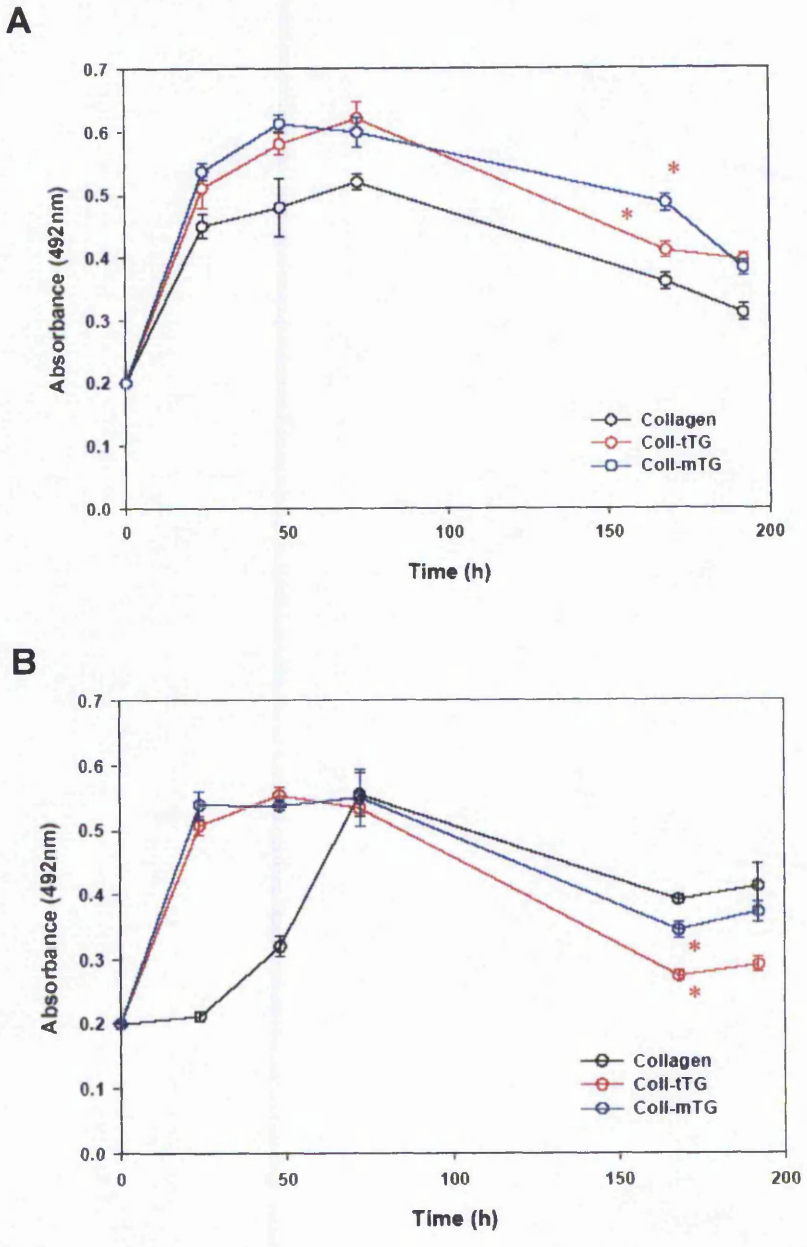


Figure 4.2.9. Proliferation of HOB cells on native and TG-treated collagen I substrates. The proliferation rates of $2x10^4$ cells HOB cells were cultured on native, (**A**) 50µg/ml TG (**B**) 100µg/ml TG treated matrices were determined using the CellTiter AQ assay (Promega, UK) as described in the Methods. Enzyme activities: tTG: 17200 Units/mg; mTG: 18700 Units/mg. Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from four independent experiments each with triplicate samples. Results obtained following treatment with TG were compared to native collagen and values corresponding to $p<0.05$ when compared with the control are represented with a *. Line/data represented by a (red) coloured * indicate $p<0.05$ at all time points.

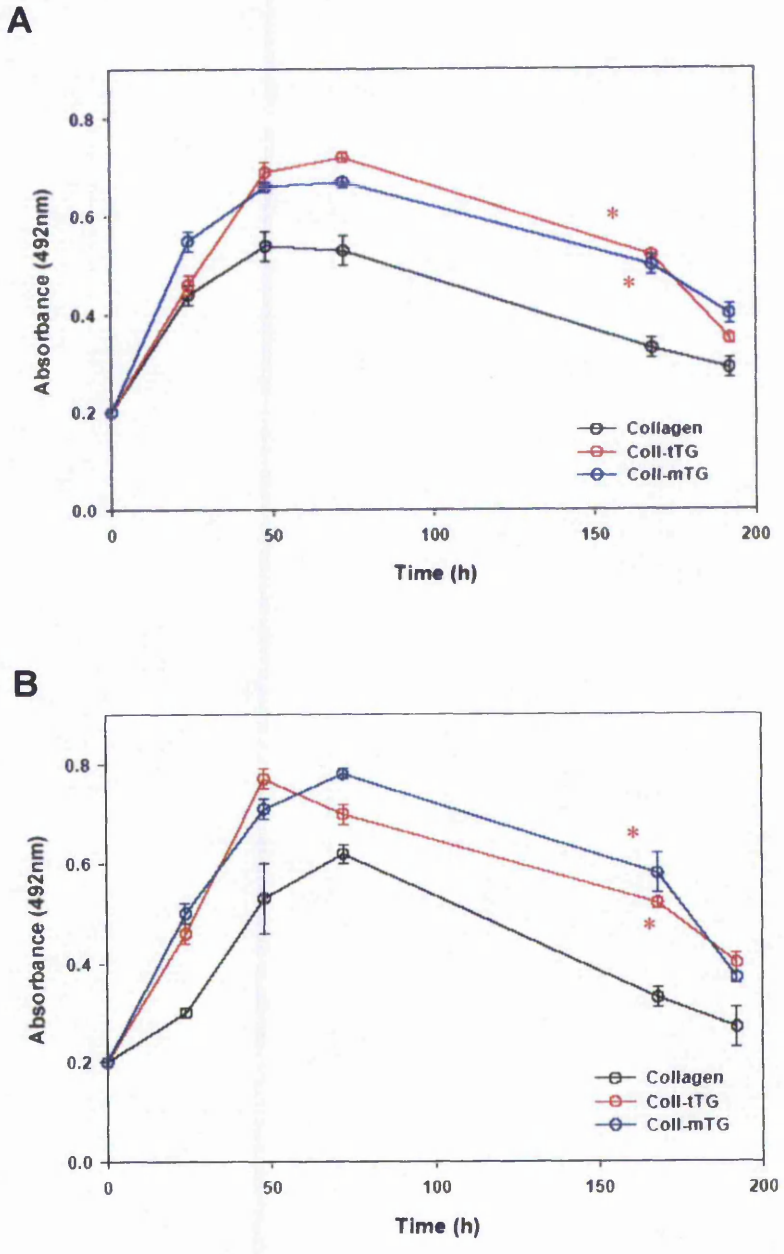


Figure 4.2.10. Proliferation of HFDF cells on native and TG-treated collagen I substrates. The proliferation rates of $2x10^4$ cells HFDF cells were cultured on native, (**A**) 50µg/ml TG (**B**) 100µg/ml TG treated matrices were determined using the CellTiter AQ assay (Promega, UK) as described in the Methods. Enzyme activities: tTG: 17200 Units/mg; mTG: 18700 Units/mg. Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from four independent experiments each with triplicate samples. Results obtained following treatment with TG were compared to native collagen and values corresponding to $p<0.05$ when compared with the control are represented with a *. Line/data represented by a (red) coloured * indicate $p<0.05$ at all time points.

4.2.5. Differentiation of HOB cells on native and TG-treated collagen substrates

The extent of how the matrices altered the differentiation profile of HOB cells was monitored by the early and late biomarkers, alkaline phosphatase (ALP) and osteopontin (OPN), respectively, as shown by Figures 4.2.11. It can be seen that increases in ALP activity occurred in both the tTG and the mTG-cross-linked collagen substrates; with the greatest increase noted with the tTG-treated collagen (Figures 4.2.11A). Furthermore, a dose-dependent relationship between the concentration of TG used to mediate the cross-linking and the increase in ALP activity of the HOB cells ($p<0.05$) can be observed. Interestingly however, with the highest concentration of mTG (250µg/ml), there appeared to be a reduction in the corresponding amount of ALP activity when compared to the same treatment with tTG. Generally, however, it can be seen that a dose-dependent relationship exists between TG concentration and the corresponding increase in the ALP activity of the HOB cells ($p<0.05$) throughout the culture period.

In the case of OPN expression, higher levels of OPN were observed in all the transglutaminase-modified collagen substrates- with the greatest increases seen with 250µg/ml of transglutaminase treatment (Figures 4.2.11B). This concentration gave rise to a corresponding two-fold increase in the level of OPN expression over the native collagen. In summary, a dose-dependent relationship between the concentration of TG and the increase in the OPN levels of the HOB cells ($p<0.05$) was observed throughout the culture period.

4.2.6. Susceptibility to apoptosis of HOB cells when cultured on native and TG-treated collagen substrates

To determine whether the TG-treated collagen matrices initiated or decreased the amount of apoptosis compared with native collagen I, the samples were assayed using the colorimetric CaspACE assay kit (Promega, UK) which monitors the caspase-3 activity; an early regulatory event in apoptosis. Figure 4.2.12 shows the amount of caspase-3 activity in the TG-treated substrates, native collagen and a positive control (1µM staurosporine). It can be seen that apoptosis is not altered when HOB cells are cultured on TG-treated collagen I matrices compared to the native collagen I.

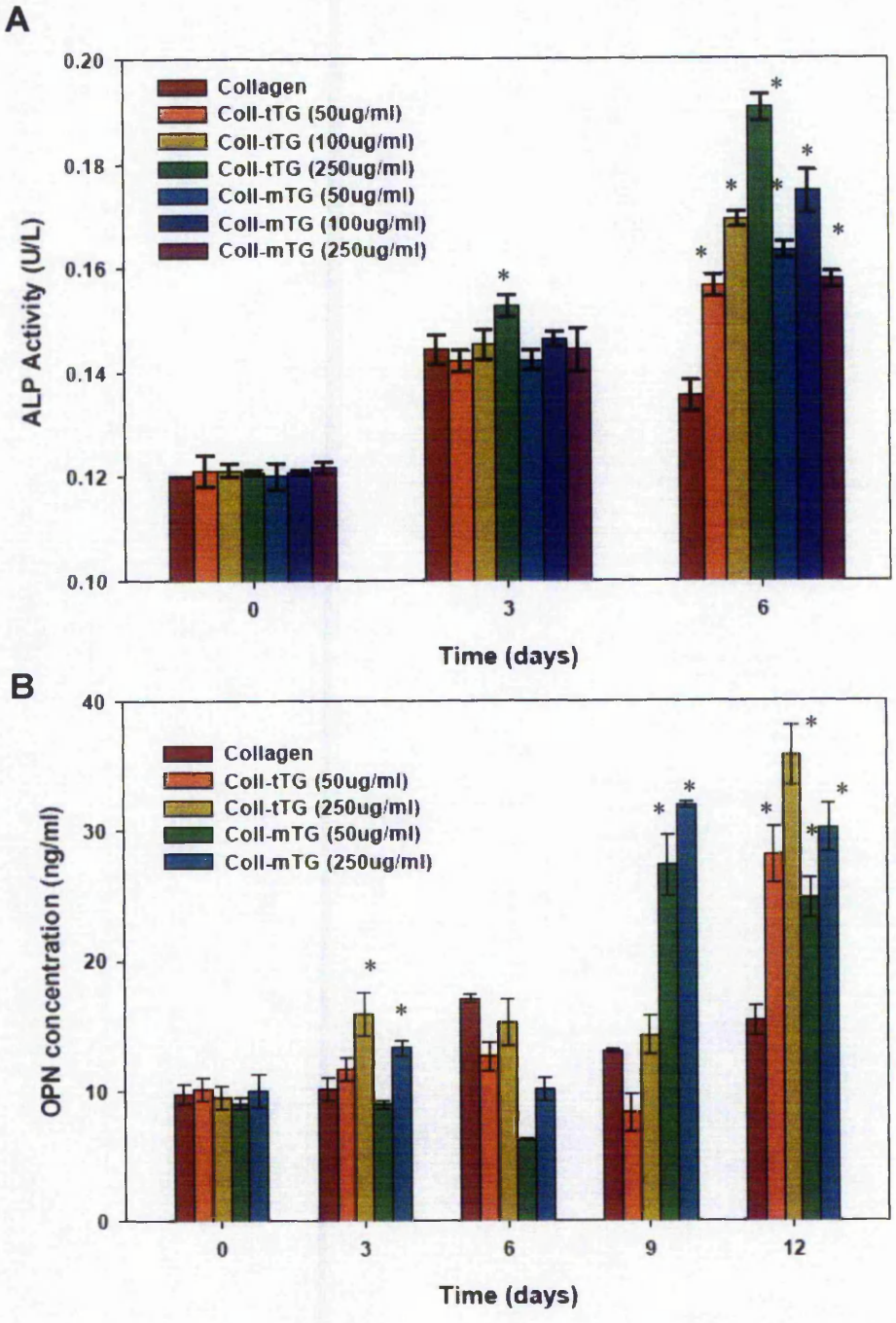


Figure 4.2.11. Differentiation of HOB cells on native and TG-treated collagen substrates. Alkaline phosphatase (**A**) and osteopontin levels (**B**) were measured as standard biochemical markers of osteoblast differentiation. Enzyme activities: tTG: 17200 Units/mg; mTG: 18700 Units/mg. Results are the mean values ±SD from three independent experiments. Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments. Results obtained following treatment with TG were compared to native collagen and values corresponding to $p<0.05$ when compared with the control are represented with a *.

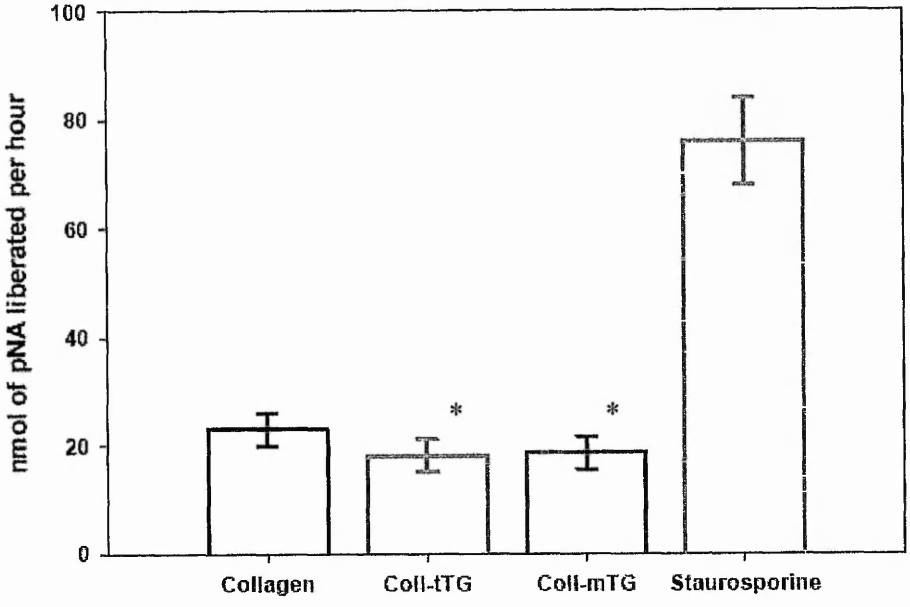


Figure 4.2.12. Caspase-3 activity of HOB cells. The extent of native and 100µg/ml TG-treated collagen matrices inducing apoptosis was determined using the CaspACE Assay system (Promega, UK) as described in the Methods. 2 x 10^5 cells/well of a 6-well plate were allowed to proliferate for 24h in a humidified-atmosphere incubator at 37°C and with 5% (v/v) CO_2. Enzyme activities of TG used to crosslink the collagen were tTG: 17200 Units/mg; mTG: 18700 Units/mg. Apoptosis in the positive control was induced using 1µM staurosporine. Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments. Results obtained following treatment with TG were compared to native collagen and values corresponding to $p<0.05$ when compared with the control are represented with a *.

4.2.7. Confirmation of MEF-WT and MEF-KO cell genotypes

For identification of the mouse embryonic fibroblast wild-type (MEF-WT) and TG-knock out variant (MEF-KO) cell lines, their genotypes were confirmed and analysed by multiplex PCR. The characteristic cellular products for each of the cell lines can be identified by the respective positive bands as shown by Figure 4.2.13.

4.2.8. Proliferation characteristics of MEF-WT and MEF-KO cells on native and TG-treated collagen substrates

To consider the potential role of (cellular) TG in "normal" cell behaviour, the proliferation characteristics of the MEF cells were monitored with the CellTiter cell proliferation assay kit (Promega, UK) at the 6 and 24-hour time-points during their culture on native and TG-treated collagen substrates. The results for the MEF-WT cell line, as seen in Figure 4.2.14A, indicate a significant increase ($p<0.05$) in proliferation when cultured on tTG-treated collagen, at the 24h time-point compared to native collagen. However, no significant difference ($p>0.05$) was identified between MEF-WT cells cultured on mTG-treated collagen substrates and those cultured on the native collagen at the same time-point. In the case of MEF-KO cells, Figure 4.2.14B, it can be seen that the cells cultured on tTG-treated collagen matrix demonstrated a significant ($p<0.05$) advantage in proliferation compared to the other matrices at the 6h time-point. However, the most striking difference can be identified between cells cultured on TG-treated collagen substrates to that of cells cultured on native collagen at the 24h time-point: cells cultured on tTG-treated collagen demonstrated ~35% increase in its proliferation characteristic, whereas the cells grown on collagen treated with mTG showed ~10% increase.

4.2.9. Confirmation of TG-mediated incorporation of peptides and fibronectin into collagen: cross- link analysis

To assess the potential of cross-linking additional synthetic growth/attachment peptides (i.e. commercial EP011-803 and EP011-804 attachment analogues, peptide-QQ, peptide-KK and fibronectin) into collagen type I using 100µg/ml of tTG or mTG was performed as previously described. Formation of the ε-(γ-glutamyl)lysine cross-linking was determined using ion-exchange after extensive proteolytic digestion and summarised in Table 4.2.2.

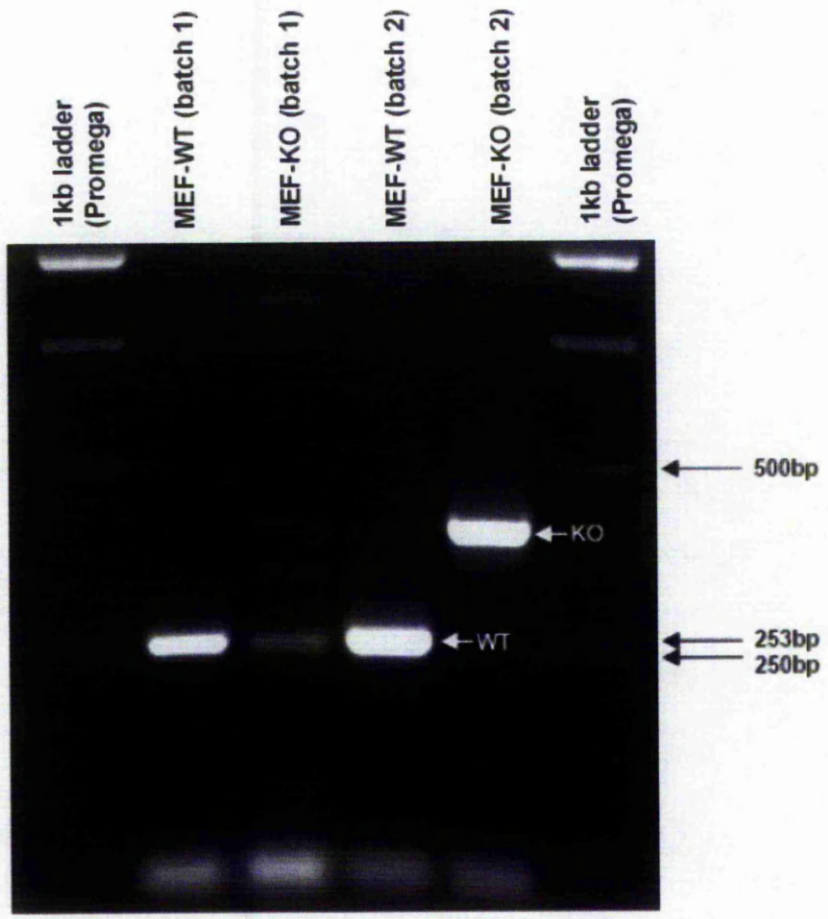


Figure 4.2.13. PCR confirmation of MEF-WT and MEF-KO cell lines. Total RNA was extracted from 2 x 10^7 cells using the RNeasy mini-kit from Qiagen (Crawley, UK). Reverse transcription (RT)-PCRs were performed with the RT-PCR One Step System (Life Technologies, Paisley, UK), using 200ng of total RNA, according to the manufacturer's instructions. The primers used for the amplification of TG-2 were TG30 (5'GACAACAACTATGGGGATGGT3') and TG9B (5'ATCATCTCGCTCTTGTTCGTC3') with running conditions set as: initial denaturation at 95°C for 5 min, cycles of 95°C for 1 min, 68°C for 1 min, 72°C for 1 min, reducing the annealing temperature each cycle by 2°C until it reached 60°C, followed by 35 cycles at a 60°C annealing temperature. PCR products were resolved on a 1.6% agarose gel and stained with ethidium bromide. Expected end-products corresponding to the neomycin resistance gene, antisense primer in intron 5 and a sense primer in intron 4 are labeled with respect to the desired MEF-WT and MEF-KO cell line.

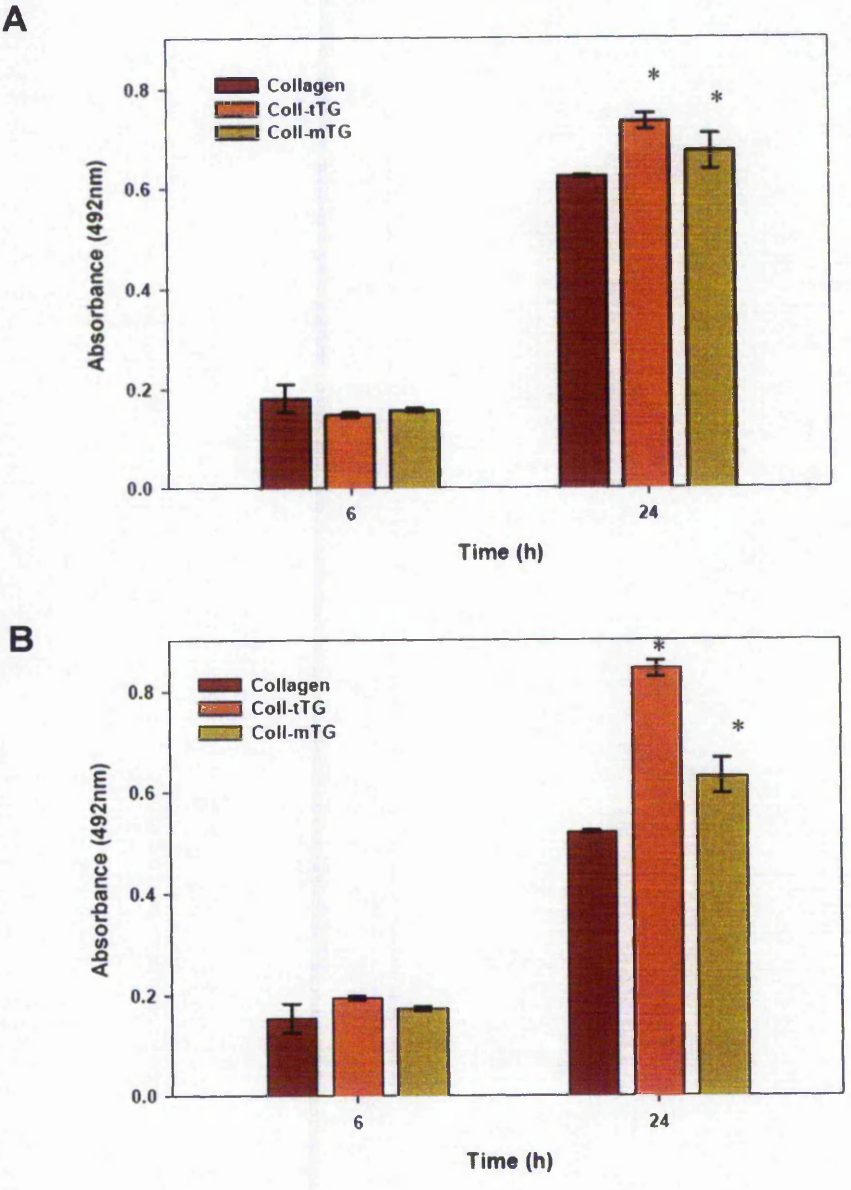


Figure 4.2.14. Proliferation of MEF-WT and MEF-KO cells on native and TG-treated collagen I substrates. The proliferation rates were determined using the CellTiter AQ assay (Promega, UK) at 6 hour and 24 hours time points for the (2 x 10^4 cells initially seeded) (**A**) MEF-WT and (**B**) MEF-KO cells cultured on native and 100µg/ml TG-treated collagen matrices (Enzyme activities: tTG: 17200 Units/mg; mTG: 18700 Units/mg). Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments each having triplicate samples. Results obtained following treatment with TG were compared to native collagen and values corresponding to $p<0.05$ when compared with the control are represented with a *.

Sample	nmol of cross-link/ mg protein sample	± relative change to native collagen*	mol cross-link/mol of collagen+
Collagen	0.26	-	0.12
Coll-tTG	0.69	2.63	0.32
Coll-mTG	0.95	3.64	0.44
Coll-tTG-803(LP)	0.58	2.21	0.27
Coll-tTG-803(HP)	0.70	2.69	0.32
Coll-tTG-804(LP)	0.76	2.93	0.35
Coll-tTG-804(HP)	0.86	3.30	0.40
Coll-tTG-QQ(LP)	1.29	4.97	0.60
Coll-tTG-QQ(HP)	1.68	6.46	0.77
Coll-tTG-KK(LP)	1.20	4.61	0.55
Coll-tTG-KK(HP)	1.26	4.84	0.58
Coll-tTG-Fn(LP)	0.49	1.90	0.23
Coll-tTG-Fn(HP)	1.02	3.90	0.47
Coll-mTG	0.95	3.64	0.44
Coll-mTG-803(LP)	0.75	2.88	0.35
Coll-mTG-803(HP)	0.78	3.00	0.36
Coll-mTG-804(LP)	0.58	2.22	0.27
Coll-mTG-804(HP)	0.67	2.57	0.31
Coll-mTG-QQ(LP)	1.58	6.07	0.73
Coll-mTG-QQ(HP)	1.09	4.19	0.50
Coll-mTG-KK(LP)	1.68	6.46	0.78
Coll-mTG-KK(HP)	1.69	6.49	0.78
Coll-mTG-Fn(LP)	0.74	2.83	0.34
Coll-mTG-Fn(HP)	0.78	3.00	0.36

Table 4.2.2. Transglutaminase mediated cross-linking of collagen type I and the incorporation of peptides and fibronectin. Collagen samples were initially prepared at 6mg/ml. Both tTG and mTG were used at concentrations of 100µg/ml (enzyme activities: tTG: 17200 Units/mg; mTG: 18700 Units/mg). The cross-linking reaction was allowed to proceed in a humidified-atmosphere incubator overnight at 37°C and with 5% CO_2. Peptide and fibronectin concentration ranged from 5 or 50µg/ml. LP, 5µg/ml; HP, 50µg/ml. Peptide EP011-803 sequence: H2N EAQ QIV PHS RNG GGR GD COOH. Peptide EP011-804 sequence: H2N GKK GPH SRN GGG RGD COOH. Peptide QQ sequence: FITC-TVQQEL. Peptide KK sequence: FITC-KKKKGY.

It can be seen that on treatment with the transglutaminases, a corresponding increase in the amount of ε-(γ-glutamyl)lysine bonds is detected. Furthermore, a greater amount of isopeptide bonds are detected with the glutamine-rich peptide when treated with tTG; whereas more of the cross-links are detected in the samples treated with mTG and the lysine-rich peptide. This result suggests that the tTG and mTG enzymes may have relatively relaxed specificities for lysine and glutamine substrates, respectively, and hence reflecting their alternative proposed cross-linking sites. On incorporating fibronectin into the collagen matrices, it can be noted that a slightly higher amount of cross-link is achieved with tTG. Additionally, increasing the concentration of FN corresponds to an increase in the amount of isopeptide bond when tTG is used. In contrast, when mTG is used to incorporate FN, a saturation point appears to have reached despite an increase in the substrate concentration.

The histogram presented as Figure 4.2.15 documents the results derived from gas chromatography analyses of the glycine residue of the TG-treated collagen and samples incorporated with the synthetic peptides. It was noted that glycine was the dominant amino acid in the commercial peptides and also allowed a selective "spiking" of the collagen samples. Following the cross-linking and incorporation process, samples were then subjected to a 6M HCl acid hydrolysis and derivatisation prior to glycine analyses. From the results, it can be deduced that incorporation of the synthetic peptides occurs based on the corresponding resultant increase of glycine residues which are present in the synthetic peptides and analogues. Interestingly, a dose-dependent response can be noted when mTG was used to incorporate the EP011-804 peptide into matrix. Increasing the concentration of peptide corresponded to an increase of glycine residue detected and suggests that more of the presented peptide (per sample) is incorporated into the substrate. Contrastingly, a significant difference ($p<0.05$) could not be identified for the EP011-803 peptide- even when the concentration of the peptide was increased by 10-fold. When tTG was used as the incorporating agent, a dose-dependent response could not be demonstrated for either of the peptides; in fact, it appeared that a saturation level was reached- despite increases of the peptide concentration. Interestingly, it appeared that no incorporation occurred with the EP011-804 peptide when used at the higher peptide concentration

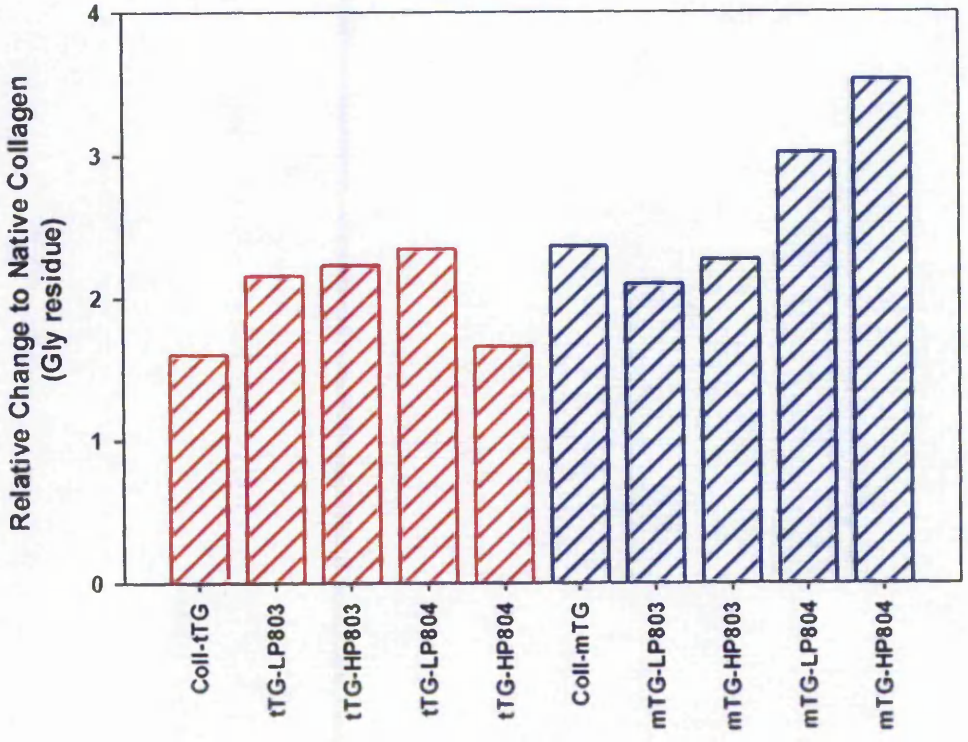


Figure 4.2.15. Glycine residue of native collagen, TG-treated collagen and collagen incorporated with synthetic peptides. Collagen samples were initially prepared at 6mg/ml. Both tTG and mTG were used at concentrations of 100µg/ml (enzyme activities: tTG: 17200 Units/mg; mTG: 18700 Units/mg). The cross-linking reaction was allowed to proceed in a humidified-atmosphere incubator overnight at 37°C and with 5% CO_2. Peptide and fibronectin concentration ranged from 5 or 50µg/ml. LP and HP refer to low percentage (5µg/ml) and HP high percentage (50µg/ml) respectively. Peptide EP011-803 sequence: H2N EAQ QIV PHS RNG GGR GD COOH. Peptide EP011-804 sequence: H2N GKK GPH SRN GGG RGD COOH. Samples were hydrolysed with 6M HCl before being derivatised using EZ:Faast™ amino acid testing kit then sampled using an Agilent Technologies 6890N Network GC system running under constant flow mode: 1:15 split injection at 250°C, 2.5µl; using helium as the carrier gas at 60kPa, with 3 kPa/min increments. The oven was programmed for 32°C/min from 110°C to 320°C, holding at 320°C for 1 minute. Detection was achieved using a FID set at 320°C.

even though a significant increase ($p<0.05$) was detected at the lower concentration. To further confirm the incorporation process, the residual fluorescence of cross-linked FITC-labelled peptides, mediated by 100µg/ml tTG or mTG treatment, into the collagen substrates was also monitored as shown by the histograms in Figure 4.2.16. It can be seen that both peptides, FITC-TVQQEL and FITC-KKKKGY, were incorporated via tTG and mTG into the matrices based on the increase of fluorescence of each sample. Interestingly, a greater amount of the FITC-TVQQEL (Figure 4.2.16A) peptide was incorporated when tTG was used, whereas more of the FITC-KKKKGY was detected in the samples treated with mTG (Figure 4.2.16B) in agreement with the previous cross-linking gas chromatography analyses (section 4.2.9).

4.2.10. Proliferation characteristics of HOB cells on TG-treated and peptide-incorporated collagen substrates

The proliferation characteristics of HOB cells were monitored on TG-treated and both the peptide-incorporated matrices (EP011-803, EP011-804; 5µg/ml and 50µg/ml concentrations) at the 6 and 24-hour culture period time-points. On treatment with 100µg/ml tTG, as seen in Figure 4.2.17A, only a small but significant improvement ($p<0.05$) in proliferation can be noted with cells cultured on the 50µg/ml EP011-803 matrix; no comparable differences can be seen with the other matrices during the short-term (6h time-point). However, during long-term (24h) culture, all the peptide-incorporated matrices induced a more apparent increase in the proliferation characteristics of the HOB cells- with optimal results provided by the 50µg/ml EP011-803 matrix. Furthermore, a dose-dependent response can be noted for each of the peptide-incorporated matrices. In the case of treatment with 100µg/ml mTG, as seen in Figure 4.2.17B, only a minor but significant improvement ($p<0.05$) in proliferation can be noted with cells cultured on the 5µg/ml EP011-804 matrix. Interestingly, a reduction of the proliferation characteristics occurred with both the 50µg/ml peptide matrices during short-term (6h time-point) culture. During the long-term culture, the majority of the peptide-incorporated matrices induced significant increases in proliferation of the HOB cells; although no significant difference could be identified on the 5µg/ml EP011-804 matrix. Optimal proliferation occurred on the EP011-803 matrices which were surprisingly comparable at both concentrations during the long-term culture time-point.

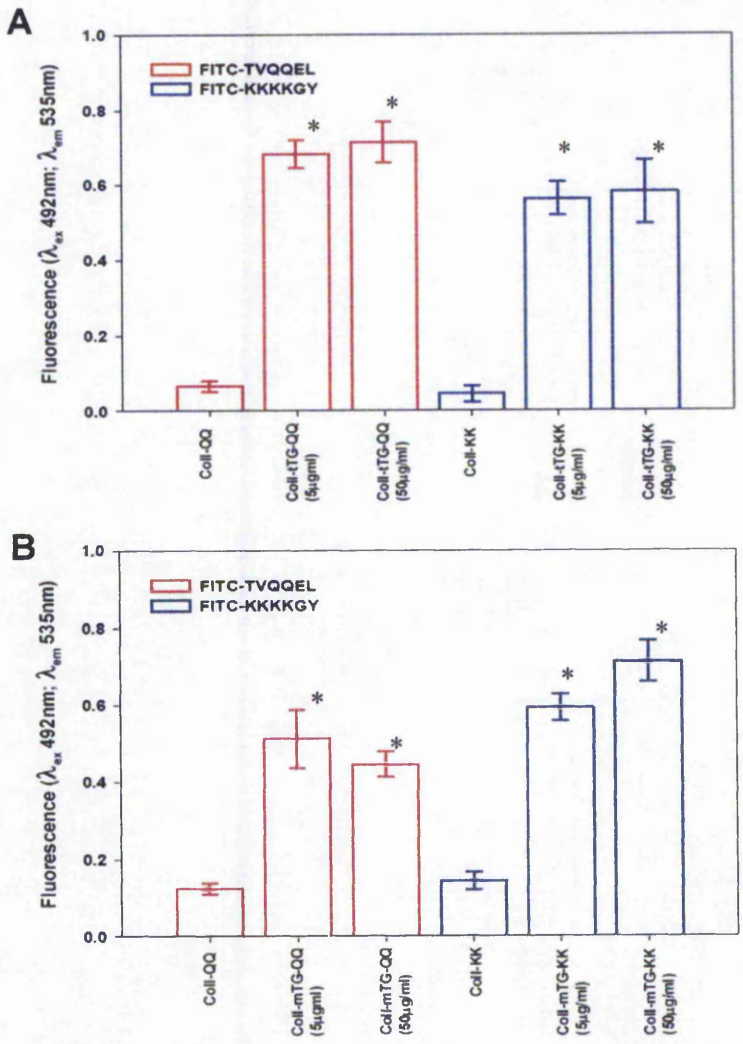


Figure 4.2.16. Detection of FITC-labelled peptides incorporated within TG-treated collagen substrates. Collagen samples were treated with either 5µg/ml or 50µg/ml of FITC-TVQQEL or FITC-KKKKGY peptide, incorporated via 100µg/ml of (**A**) tTG and (**B**) mTG (enzyme activities: tTG: 17200 Units/mg; mTG: 18700 Units/mg). Following incubation overnight in a humidified-atmosphere incubator at 37°C and with 5% (v/v) CO_2, samples were, washed and treated to with microbial collagenase and trypsin as described in the Methods. A 75µl aliquot of supernatant was then removed and the fluorescence measured using an excitation and emission wavelength of 492nm and 535nm, respectively. LP and HP refer to low percentage (5µg/ml) and HP high percentage (50µg/ml) respectively. Peptide QQ sequence: FITC-TVQQEL. Peptide KK sequence: FITC-KKKKGY. Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments each having triplicate samples. Results obtained following treatment with TG and peptides were compared to native collagen and peptides and values corresponding to $p<0.05$ when compared with the control are represented with a *.

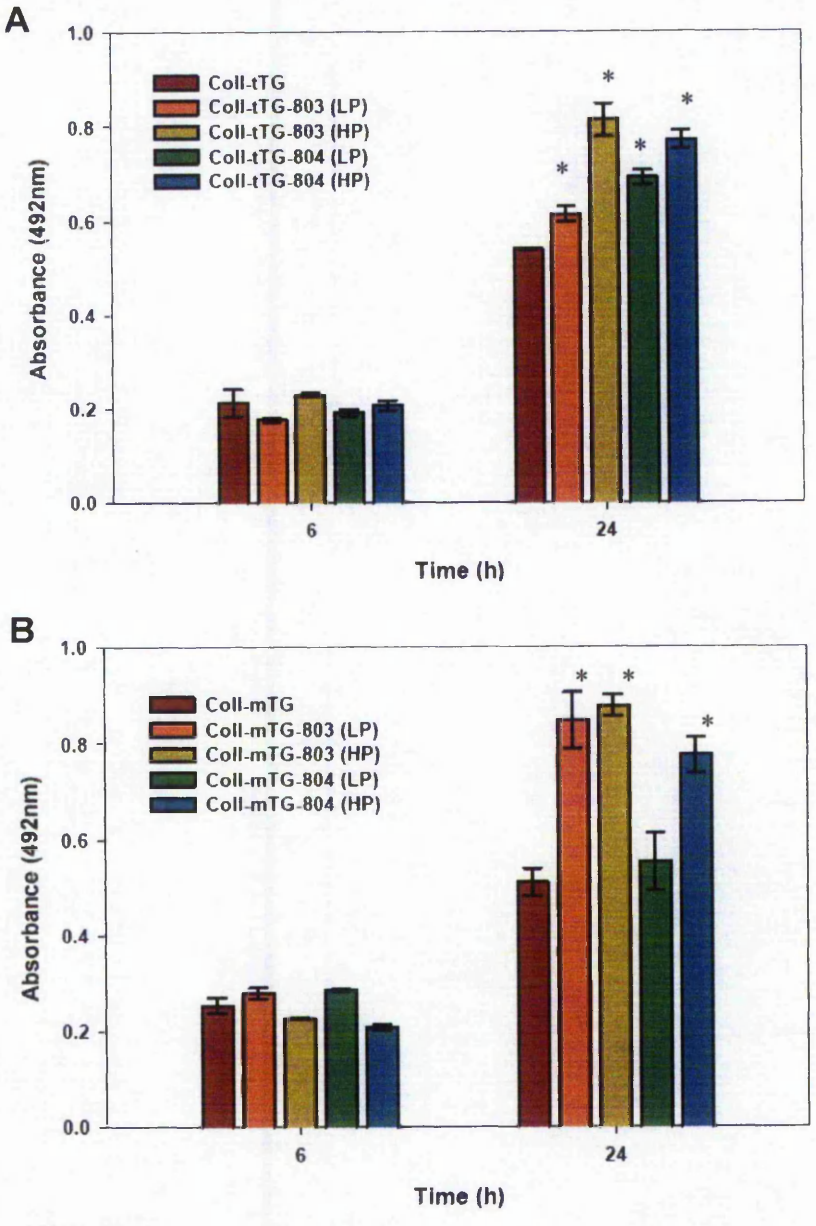


Figure 4.2.17. Proliferation of HOB cells on TG-treated and peptide incorporated collagen substrates. Proliferation rates of 2 x 10^4 HOB cells cultured on 100µg/ml tTG-treated (**A**) and 100µg/ml mTG-treated (**B**) collagen matrices were determined using the CellTiter AQ assay (Promega, UK). Enzyme activities: tTG: 17200 Units/mg; mTG: 18700 Units/mg. Peptide EP011-803 sequence: H2N EAQ QIV PHS RNG GGR GD COOH and Peptide EP011-804 sequence: H2N GKK GPH SRN GGG RGD COOH. LP and HP refer to low percentage (5µg/ml) and HP high percentage (50µg/ml) respectively. Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments each having triplicate samples. Results obtained following treatment with TG and peptides were compared to native collagen-TG. Values corresponding to $p<0.05$ when compared with the control are represented with a *.

4.2.11. Attachment and spreading characteristics of HOB and HFDF cells on TG-treated and fibronectin-incorporated collagen substrates

Fibronectin is the most documented ECM protein that has been successfully exploited in the improvement of the biocompatibility of a biomaterial and as such, Figure 4.2.18 shows the cell-attachment characteristics of HOB and HFDF cells cultured on 100μg/ml TG-treated and 5μg/ml or 50μg/ml fibronectin-incorporated into the collagen matrices at the 1h and 3h time-points. A negative control involved the use of collagen plus the corresponding concentration of FN without the TG enzyme. It can be seen that for the HOB cells, at the 1h time-point, an increase in the number of cells attaching occurs on the FN-incorporated matrices with the only exception being the 50μg/ml tTG-FN matrix (Figure 4.2.18A). However, at the 3h time-point, the incorporation of FN into the matrices consequently increases the number of cells attaching to the modified matrices compared to the original TG-treated only substrates. Furthermore, a dose-dependent response can be seen with the mTG-FN matrices. In the case of HFDF cells, at the 1h time-point, significant differences can be noted in all the FN-modified matrices compared to the TG-treated only counterparts (Figure 4.2.18B). Furthermore, a dose-dependent response can be seen with both tTG-FN and mTG-FN matrices. Interestingly, the maximum number of attached HFDF cells is reached by all the FN-incorporated matrices at the 3h time-point.

Figures 4.2.19A and B show the short-term (1h and 6h time points) spreading characteristics of the HOB and the HFDF cells, respectively, when cultured on 100μg/ml TG-treated and 5μg/ml or 50μg/ml fibronectin-incorporated collagen matrices. It can be observed that significant enhancements of the spreading characteristics are noted on the 5μg/ml FN-tTG substrates for both the HOB and HFDF cells ($p<0.05$) at the two time points. However, in the case of the 50μg/ml FN-tTG matrix, at both times points, a decrease of the spreading characteristics is noted when compared to the normal TG-crosslinked substrates- although this does not happen with the lower concentration FN-tTG matrix- which, interestingly, provides the optimum spreading characteristics.

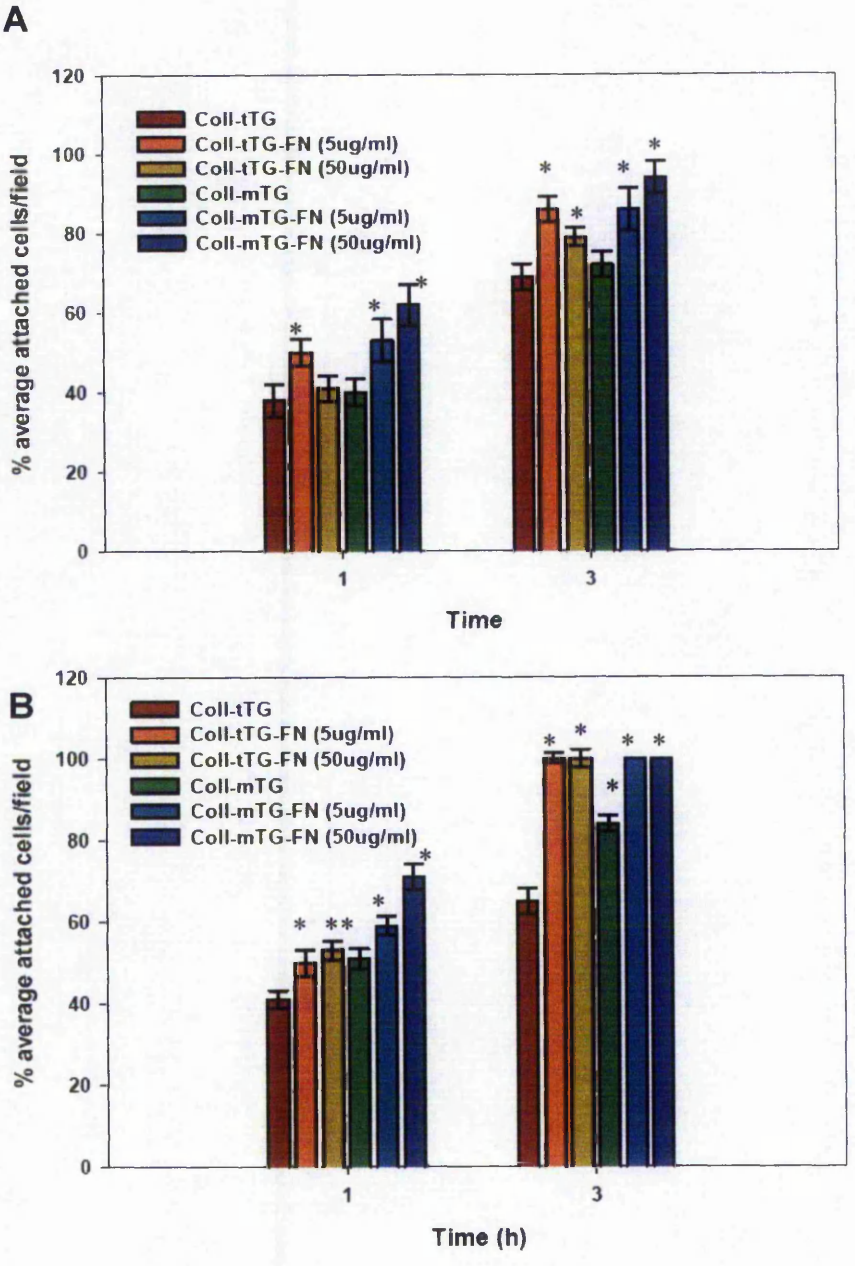


Figure 4.2.18. Attachment of HOB and HFDF cells on TG-treated and fibronectin (FN) incorporated collagen I substrates. $2x10^4$ (**A**) HOB cells and (**B**) HFDF cells were cultured on 100µg/ml TG-treated and 5µg/ml or 50µg/ml FN-incorporated collagen matrices at the 1h and 3h time-points. Enzyme activities: tTG: 17200 Units/mg; mTG: 18700 Units/mg. Attachment is expressed as a percentage of the total number of cells after 6h. Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments each having triplicate samples. Results obtained following treatment with TG and FN were compared to native collagen-TG. Values corresponding to $p<0.05$ when compared with the control are represented with a *.

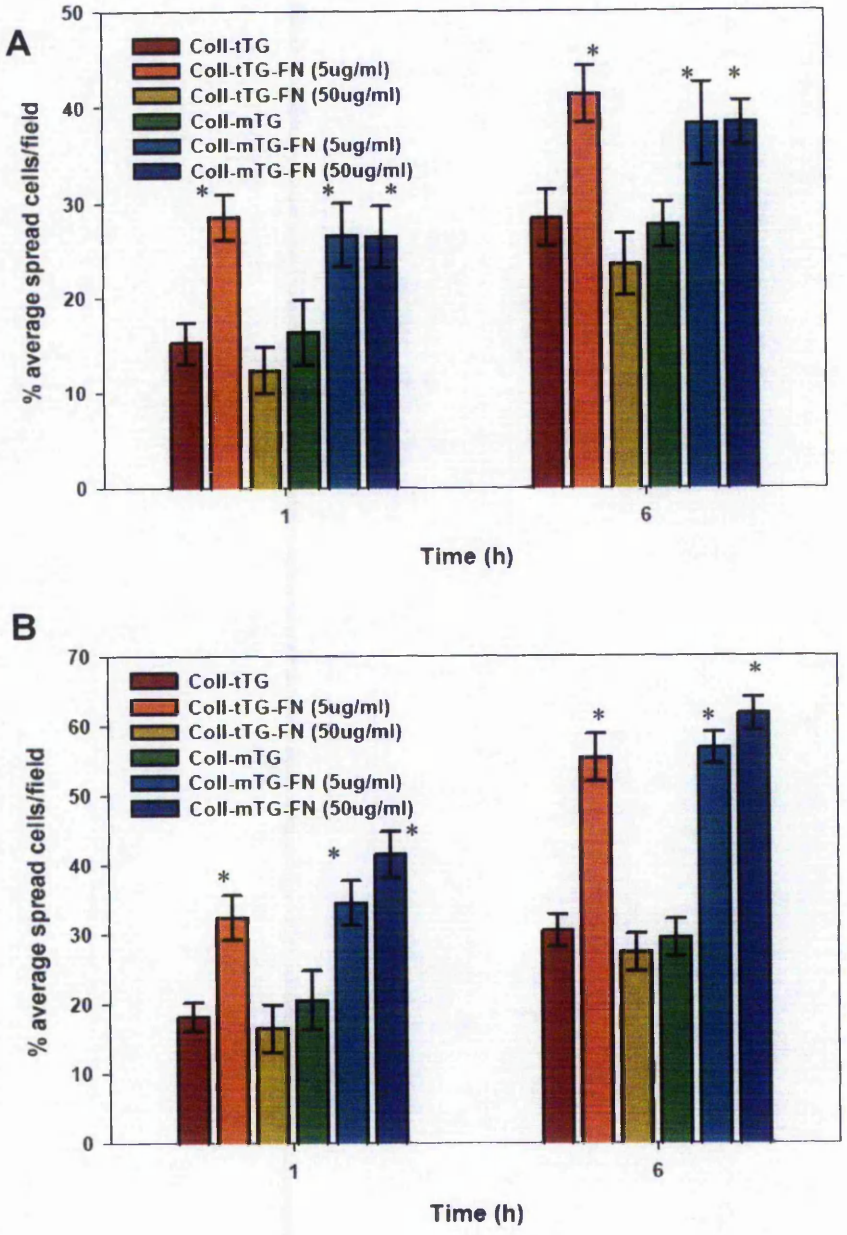


Figure 4.2.19. Spreading of HOB and HFDF cells on TG-treated and fibronectin (FN) incorporated collagen substrates. $2x10^4$ (**A**) HOB cells and (**B**) HFDF cells were cultured on 100µg/ml TG-treated and 5µg/ml or 50µg/ml FN-incorporated collagen matrices at the 1h and 3h time-points. Enzyme activities: tTG: 17200 Units/mg; mTG: 18700 Units/mg. Spreading is expressed as a percentage of the total number of cells in the field of view. Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments each having triplicate samples. Results obtained following treatment with TG and FN were compared to native collagen-TG. Values corresponding to $p<0.05$ when compared with the control are represented with a *.

4.2.12. Proliferation characteristics of HOB and HFDF cells on TG-treated and fibronectin-incorporated collagen substrates

The proliferation characteristics of both the HOB and HFDF cells were monitored on TG-treated and both the FN-incorporated matrices (5µg/ml and 50µg/ml FN concentrations) at the 6 and 24-hour culture period time-points. In the case of the HOB cells (Figure 4.2.20A), it can be seen, with the exception of the 50µg/ml FN-tTG matrix, that the matrices incorporated with fibronectin induce an increase of the proliferation rates of the HOB cells at the 6h time-point. At the 24h time-point, the same profile is apparent- with a dose-dependent response being prominent for the mTG-treated matrices. In the case of HFDF cells (Figure 4.2.20B), all the matrices with fibronectin incorporated increase the amount of proliferation at the 6h time-point. This behaviour and profile is maintained at the 24h time-point by the majority of the matrices, the 50µg/ml FN-tTG substrate appears to cause a decrease in the proliferation characteristics of the HFDF cells- to an extent that it is even below that of the tTG-treated collagen substrate.

4.2.13. Migration characteristics of HFDF cells native, tTG-treated and fibronectin-incorporated collagen substrates

The migration of HFDF cells in the native, tTG-crosslinked and 50µg/ml FN-tTG incorporated collagen substrates was assessed using the 'agar drop' assay as described in the Methods section. In short, this assay involves the migration of cells out of the cell containing agar drop placed onto the collagen substrates over a defined time period, as shown by the images in Figure 4.2.21 and the results summarised in Table 4.2.3. On summarising, it was noted that the cell migration appeared to occur faster, with a greater number of cells, migrating out on both the tTG-treated and FN-incorporated matrices. The migratory characteristics appeared to be optimal on the FN-tTG-treated collagen substrate and achieved 100% migration.

4.2.14. Resistance of TG-treated and fibronectin-incorporated collagen substrates to HOB and HFDF cell mediated degradation

Figure 4.2.22 documents the extent of degradation of the TG-treated and FN-incorporated matrices following 72h of culture with both HOB and HFDF cells and determined by measuring the protein remaining in the matrix after

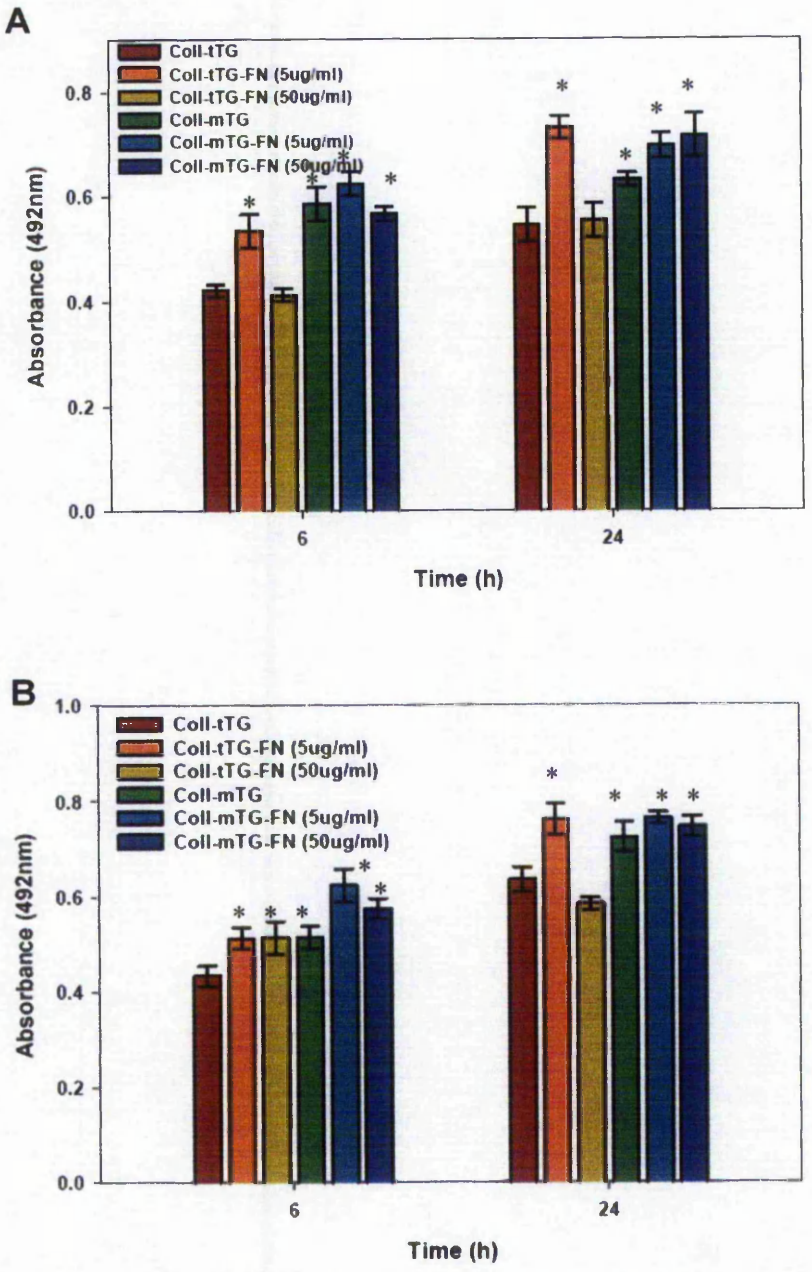


Figure 4.2.20. Proliferation of HOB and HFDF cells on TG-treated and fibronectin (FN) incorporated collagen substrates. The proliferation rates of $2x10^4$ (**A**) HOB and (**B**) HFDF cells cultured on 100µg/ml TG-treated and 5µg/ml or 50µg/ml FN-incorporated collagen matrices were determined using the CellTiter AQ assay (Promega, UK). Enzyme activities: tTG: 17200 Units/mg; mTG: 18700 Units/mg. Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments each having triplicate samples. Results obtained following treatment with TG and FN were compared to native collagen-TG. Values corresponding to $p<0.05$ when compared with the control are represented with a *.

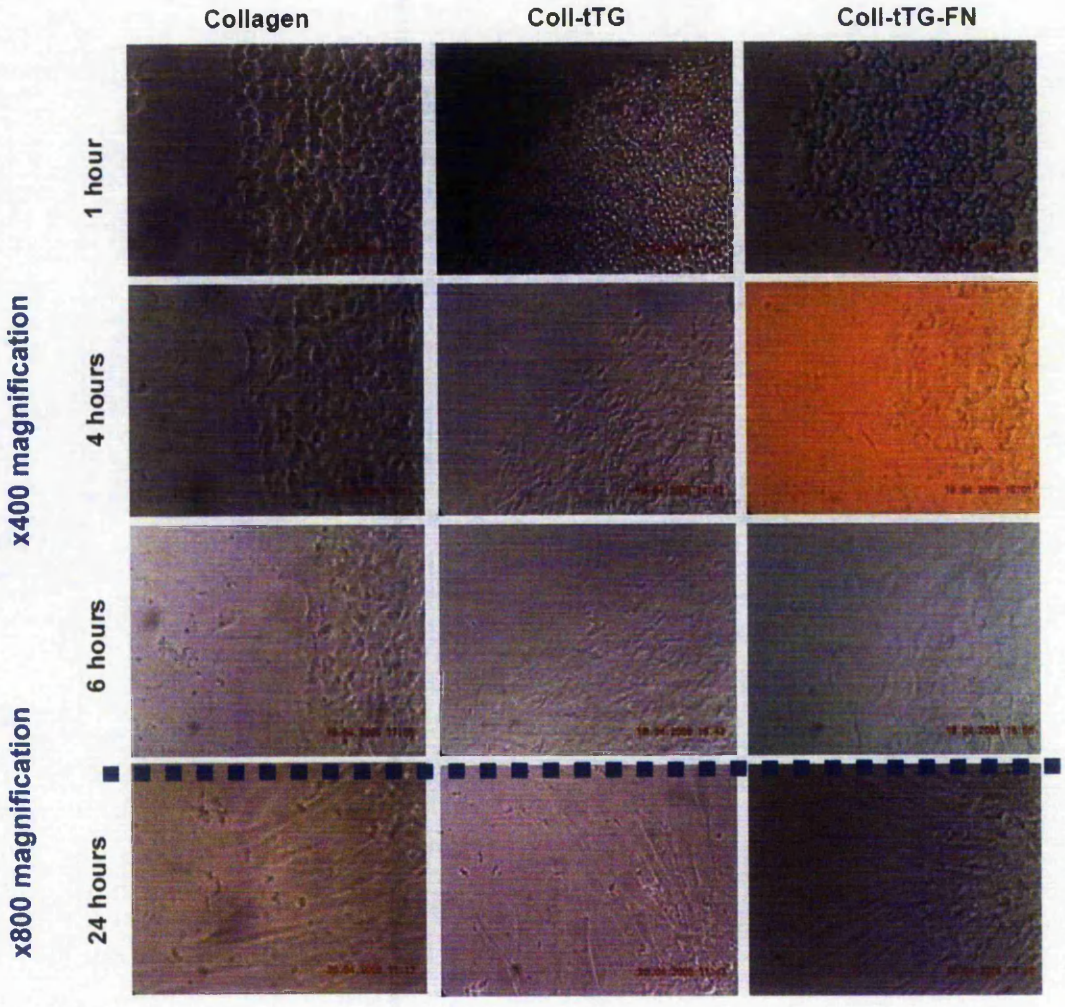


Figure 4.2.21. Migration of HFDF cells on native and TG-treated collagen substrates. The migration of HFDF cells in the native, tTG-crosslinked and 50µg/ml FN-tTG incorporated collagen substrates was assessed using the 'agar drop' assay as described in the Methods section. Images at selective time points monitoring migration of HFDF cells cultured on native, 100µg/ml TG-treated and 50µg/ml FN-incorporated collagen matrices using the 'agar drop' as described in Methods. Enzyme activities: tTG: 17200 Units/mg

Time	Collagen	Collagen-tTG	Collagen-tTG-FN
1 hour	-	-	-
4 hours	+	++	++
6 hours	++	+++	++++
24 hours	40%	60%	100%

Table 4.2.3. Qualitative peripheral migration of HFDF cells on native and TG-treated collagen. Images at selective time points monitoring migration of the HFDF cells cultured on native, 100µg/ml TG-treated and 50µg/ml FN-incorporated collagen matrices using the 'agar drop' as described in Methods were compared. Enzyme activities: tTG: 17200 Units/mg

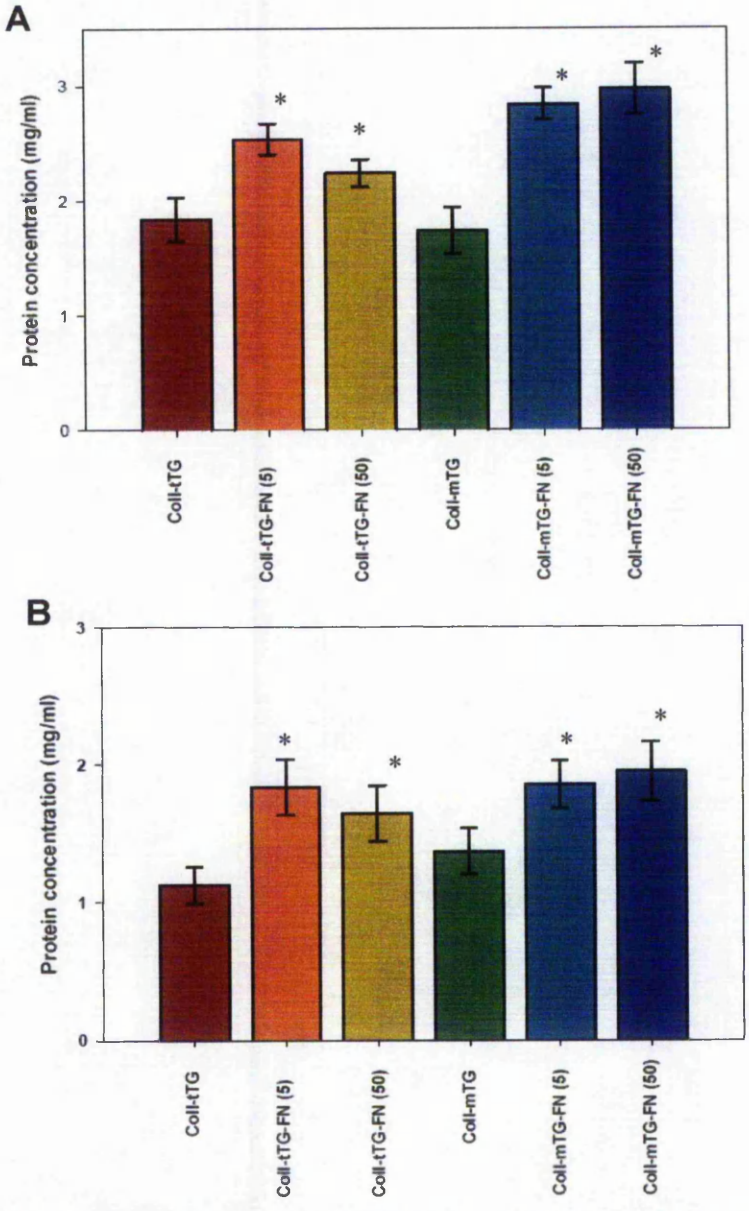


Figure 4.2.22. Degradation of TG-treated collagen and fibronectin (FN) incorporated collagen substrates by HOB and HFDF cells. 2 x 10^4 cells were cultured on 100µg/ml TG-treated and 5µg/ml and 50µg/ml FN-incorporated collagen matrices for 72 hours. Cells were removed with sodium deoxycholate and matrices treated to proteolytic action with microbial collagenase and trypsin. Enzyme activities: tTG: 17200 Units/mg; mTG: 18700 Units/mg. The residual solubilised protein was measured using the Lowry assay as described in the Method section. Initial collagen levels were 3mg/ml. Residual protein concentration of matrices cultured with (**A**) HOB and (**B**) HFDF cells. Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments each having triplicate samples. Results obtained following treatment with TG and FN were compared to native collagen-TG. Values corresponding to $p<0.05$ when compared with the control are represented with a *.

solubilisation of the residual protein by proteolytic digestion. The concentration of the residual protein in the collagen matrices following HOB cell culture can be seen in Figure 4.2.22A. These results demonstrate a significantly reduced degradation occurring in the FN-incorporated matrices ($p<0.05$). The FN-mTG-matrices demonstrated the greatest resistance to HOB-cell-mediated degradation with negligible degradation following at the 72h culture point. A similar degradation profile can be seen in the matrices cultured with HFDF cells, although the susceptibility to degradation of these substrates was of a significantly greater extent to that of the HOB-cell-mediated variants ($p<0.05$) as seen in Figure 4.2.22B.

4.3. DISCUSSION

During culture of HOB and HFDF cells, it can be seen that native collagen type I, itself, is an adequate substrate for the maintenance, growth and survival of cells, in agreement with the many years of successful exploitation of collagen in numerous applications [Mokonjimobe *et al.*, 1992; Marriott *et al.*, 1991; Nusgens *et al.*, 1984]. Interestingly, however, the TG-cross-linked collagen matrices demonstrate enhanced attachment, spreading and proliferation characteristics of the cells. More importantly, the long-term growth of these cells was also maintained on the modified matrices without the induction of any apoptosis or necrotic characteristics during the culture period. Furthermore, the HOB cells were found to differentiate more rapidly following culture on these modified substrates as demonstrated by the corresponding increases in ALP activities and the earlier appearance/induction of OPN- both being biomarkers of bone differentiation and turnover [Swaminathan, 2001].

The data presented suggest that the TG-treated matrices may be eliciting a different or premature cellular response compared to the untreated native collagen. Although it may be suggested that the observed cellular behavior results from the direct contact/interaction between the cells and the enzyme, reference to the western blots and SDS-PAGE for detection of residual TGs in the matrices, indicated no enzyme remained entrapped in the matrices. This eliminates the possibility of a direct enzyme-mediated mechanism whereby tTG is acting as an independent cell adhesion protein [Verderio *et al.*, 2000 and 1998]. Therefore, it must be postulated that the observed cellular behaviour was as a direct effect of the TG-modified matrices. Furthermore, the finding that both the mammalian and microbial enzymes alter the biological characteristics of collagen type I in the same manner, indicates that cross-linking is essential since it is unlikely that the mTG can also act as a cell adhesion protein given the large biochemical and physical differences [Motoki *et al.*, 1992]. It is therefore anticipated that treatment of collagen type I with the TGs lead to a modified collagen that exposes new or cryptic cell binding sites- possibly mimicking the natural remodelling/healing processes experienced *in vivo*. Another feasible explanation may be based on the fact that the cells are in contact for a longer time period with the TG-treated collagen (interpreted from the relative increase in proteolytic resistance of the matrices) and this increase in contact-time may provide/allow an enhancement of the required signalling for growth and survival of the cells and, in the case of the HOB cells,

actuate additional or delayed cell-ECM signalling necessary for differentiation [Mizuno *et al.,* 2000]. However, this scenario would only be applicable in the simplest instance: although evidence points out that "critical" (integrin) binding leading to successive cell signalling pathways occurs during the earlier stages of the cell adhesion process [Walter *et al.*, 2006; Codogno *et al.,* 1987; Horwitz *et al.,* 1985], other non-integrin dependent adhesion processes become more apparent after several hours following the initial attachment of cells [Basson *et al.,* 1990; Rao *et al.,* 1983] and both factors must be adopted for complete cellular behaviour.

Current literature indicates that the MMP-expression profile of cells changes in response to the different environmental stimuli that they are subjected to [Kahrai and Saarialho-Kere, 1999]. In this investigation, it was found that HFDF cells expressed a typical induction of collagenases following growth on native type I collagen compared to growth on standard tissue culture plastic. Interestingly, however, the MMP expression profile of HFDF cells was found to alter when cultured on the TG-cross-linked collagen substrate. It has been documented that the main substrate of MMP-1 is collagen type I [Daniels *et al.*, 2003] and, hence, it is possible that this alteration in active MMP-1 accounts for the increased resistance of cross-linked collagen to cellular degradation- although such a hypothesis would contradict the findings from the *in vitro* degradation experiments in Chapter 3 which demonstrated increased resistance to purified MMP-1 and MMP-8. Furthermore, it has been reported that MMP-1 synthesis and activation by fibroblasts is induced in order to initiate tissue restoration in response to the disruption of a 3-D *in vitro* culture system [Abe *et al.,* 2001]. During the inhibition of HFDF cell-mediated degradation using the GM6001 inhibitor (a broad range inhibitor of MMPs including MMP-1, -2, -8 and -9 [Galardy *et al.,* 1994]), the results further imply that the matrix breakdown is as a result of MMP-mediated degradation rather than as a by-product of manual handling, thermal instability or other physiological processes that may cause denaturation of the substrate e.g. pH and time. Evidence also documents the growth of cultured fibroblasts on type I collagen results in the secretion of active MMP-2 [Guo and Piacentini, 2003; Kessler *et al.,* 2001; Zigrino *et al.,* 2001].

The large increases of MMP-2 and MMP-9 as detected suggest that an "alternative" substrate may be present. It is known that the expression of

MMP-9 is a highly regulated process, whereas MMP-2 maintains a rather general expression in its latent form but its activation is tightly controlled- only occurring in response to the presence of a 3-D substratum [Gilles *et al.*, 1997; Azzam and Thompson, 1992]. This suggests the need for the presence of a gelatin or similar substrate. However, it is interesting to note that the induction and activation of MMP-2 has not been reported on either denatured collagen type I [Maquoi *et al.*, 1998; Azzam *et al.*, 1993] or on the non-fibril forming type IV collagen [Ruangpanit *et al.*, 2001] but only on the native triple-helical structure collagen [Zigrino *et al.*, 2001; Tomasek *et al.*, 1997; Strongin *et al.*, 1993]. Thus, induction of the MMP-2 activation process appears to require specific structures rather than general features shared amongst the collagen superfamily, such as the triple-helical structure or the repeating Gly-X-Y sequence [Ruangpanit *et al.*, 2001].

We have demonstrated that the physical and mechanical properties of the TG-treated matrices have changed significantly. It seems plausible that these enhanced properties are as a direct consequence of the presence/increase of ε-(γ-glutamyl)lysine isopeptide bonds between the α1 and α2 collagen chains, via the TG-mediated cross-linking reaction. In accordance with the literature, these observations would indicate that at least one of the cross-linking sites must be located around the vicinity of the MMP-1 specific target site, the (Gly_{775}-Leu/Ile_{776}) residues. This would explain both the increased resistance to purified MMP-1 and would also account for the observed changes in the physical characteristics of the collagen- as it is known that this is the part of the fibril that possesses the greatest flexibility. Moreover, the resultant enhanced strength, close proximity of fibrils (inferred from AFM and turbidity assay) and reduced flexibility of the collagen would coincide with the observations regarding the substrate/network contraction exerted by cells and the corresponding MMP-1 regulation. It has been shown that MMP-1 is down-regulated when cultured on TG-treated collagen matrices and, thus, it seems likely that the newly modified matrices are inherently recognised by the cells as a "pre-contracted tissue-like" substrate and, hence, does not require MMP-1 for tissue remodelling purposes. It has also been shown that up-regulation of MMP-1, 2 and 3 occurs when fibroblasts are localised in the non-aligned strain zone of a mechanical tissue culture systems [Mudera *et al.*, 2000]- highlighting the fact that mechanical loading and stress orientation may influence matrix remodelling via cellular protein expression. This phenomenon can be related

to the AFM results whereby the micro and macro-structural differences may have possibly elicited a cellular response based on the "direction" of strain imposed on the cultured cells. However, one could argue that the cells may not recognise the TG-treated matrices as collagen or gelatin and hence-wise, express a massive "cocktail" of MMPs in a futile attempt to break the substrate and allow the remodelling of a more favourable environment to take place. However, as the expression of MMPs is tightly regulated and controlled, it seems unlikely that the expression was a "random" cocktail. Furthermore, the enhanced cell behaviour; spreading, attachment, proliferation would not be demonstrated as the additional metabolic loading required to produce the wide range of additional proteases would inadvertently "slow" the cells down.

To further highlight the point that the TG-cross-linked matrices may be presenting a more favourable environment or, an environment that mimics an *in vivo* situation; cells derived from mice lacking tTG were utilised. In agreement with the previous results for the HOB and HFDF cells, a similar pattern can also be seen for the MEF-WT and MEF-KO cell lines; enhancement of the proliferation profiles occurs and may be directly attributed to the presence of the ε-(γ-glutamyl)lysine bonds or, via the secretion of the tTG enzyme itself, which is then used to cross-link the substrate or mediate key cellular processes further downstream leading to the enhanced proliferation observed for the MEF-KO cells. Moreover, it is interesting to note that tTG elicits the greatest (positive) response as opposed to the microbial-sourced enzyme. Thus, it may be postulated that the TG-treated collagen may be mimicking part of the natural remodelling processes with which the MEF-KO cells find more favourable or, simply, because the enzyme is derived from a mammalian source.

On incorporating synthetic peptides and fibronectin into the collagen matrices, it can be seen that additional ε-(γ-glutamyl)lysine bonds are detected (i.e. formed) suggesting that these molecules are further cross-linked/incorporated in the collagen structure. It is interesting to note that a "self-imposed" limit is attained in terms of the amount of isopeptide bonds formed (i.e. no major change in the formation of further cross-links became apparent) - even when the concentration of peptide or fibronectin was increased 10-fold (LP; 5μg/ml and HP; 50μg/ml). This observation suggests that a saturation point for the incorporation may exist and have been reached, i.e. all the possible cross-

linking sites have been fully exploited. Interestingly, the optimum incorporation of the glutamine-rich peptide (TVQQEL) was achieved when tTG was used as the biocatalyst. Conversely, a greater amount of the lysine-rich peptide (KKKKGY) was incorporated when mTG was used to catalyse the reaction. This evidence further confirms the hypotheses that these enzymes may have different proposed cross-linking sites or, that tTG and mTG may have relatively relaxed specificities for glutamine or lysine residues, respectively. Moreover, it is interesting to note that more ε-(γ-glutamyl)lysine bonds are detected when the shorter peptide sequences are used (i.e. the FITC-peptides) compared to the longer 803 and 804 peptides and, indeed, the full fibronectin protein. This observation, in itself, suggests that the length/size of the incorporating molecule may be critical in the subsequent ability to allow additional cross-links by means of preventing or blocking additional cross-link sites or modulating an inhibitory structural conformation.

During this investigation, it appeared that when the synthetic peptides or fibronectin were grafted on to the collagen, an increase/enhancement in cell behaviour and characteristics were observed. However, this behaviour only occurred when the lower, 5μg/ml, concentration of peptide or FN was used. In contrast, when the higher (50μg/ml) concentration was incorporated, there appeared to be a decrease in the cell characteristics- in some cases, an inhibitory effect could be observed. Similar observations have been described with osteoblasts on PMMA surfaces coated with RGD peptides [Kessler *et al.*, 2000] and with fibroblasts cultured on mTG-modified gelatin that incorporated fibronectin [Ito *et al.*, 2002]. A possible explanation would be that the excess synthetic peptides/fibronectin were entrapped within the collagen matrix and, via degradation or by simple diffusion, were released into the external environment and contributed to an inhibitory effect. However this explanation seems unlikely as the data provided by both the ion-exchange and gas-chromatography suggests a dose-dependent relationship and, in some instances the cells do exhibit enhanced growth characteristics with the higher concentration of peptide/fibronectin. However, the conclusion thus so far drawn must be treated with care as the inability to distinguish the exact cross-linkage that occurs (i.e. between TG-mediated collagen cross-linking and collagen-peptide/collagen-FN incorporation) requires further clarification.

CHAPTER 5:
CELLULAR RESPONSE

CHAPTER 5: CELLULAR RESPONSE

5.1. INTRODUCTION

In the previous chapters, it was confirmed that the TG-mediated cross-linking of native collagen improves its physical and mechanical properties. In addition, the modification further improved the biocompatibility of the collagen by enhancing cell spreading, attachment, proliferation, migration and differentiation of several cell lines. Furthermore, long-term cell growth was maintained without the induction of apoptosis or premature cell death. The biological response of the cells to TG-modified collagen was also characterised and demonstrated differences in many cell-ECM interactions including MMP expression. It was also demonstrated that further cell enhancements to the TG-modified collagen may be performed via the incorporation of synthetic (adhesion/growth) peptides and/or natural ECM proteins, such as fibronectin.

Cell-ECM interactions play important roles in many biological processes and these interactions generate the intracellular signals important for growth, survival and migration [Schlaepfer *et al.*, 1999]. Much of the cellular effects of the ECM are mediated by the integrins, a family of heterodimeric transmembrane receptors that couple components of the ECM with the cellular actin cytoskeleton in the regulation of cell shape and tissue architecture [Ruoslahti and Reed, 1994; Hynes, 1990; Burridge *et al.*, 1988]. The sites of integrin binding to the ECM are termed focal adhesion contact points and such structures have been implicated to play an important role in mediating the adhesion of cells to their substrate as well as the orchestration of cell-ECM signalling, transduction and communication [Aplin *et al.,* 1998; Giancotti, 1997; Schwartz *et al.*, 1995; Burridge *et al.,* 1988]. However, since the integrins receptors do not posses catalytic activity, signal transduction via ECM-integrin interactions occurs through the clustering and activation of a number of familiar signalling pathways/proteins which include the protein-tyrosine kinases (PTKs), a variety of nonreceptor PTKs such as Abl, Syk, FAK and Src-family PTKs, small GTP-binding proteins and serine/threonine and lipid kinases [Parsons, 1996; Guan, 1997; Hanks and Polte, 1997; Schwartz *et al.,* 1995]. All available information suggests that FAK is at a crossroad for multiple signalling pathways and an overview of what is known is shown in Figure 5.1.1.

These signalling pathways are known to regulate the variety of cellular functions including spreading, proliferation, apoptosis and migration and defining. Hence, defining and characterising these pathways is central to the understanding of the mechanism of integrin-regulated cellular functions [Assoian, 1997; Lauffenburger and Horwitz, 1996; Ruoslahti and Reed, 1994.]

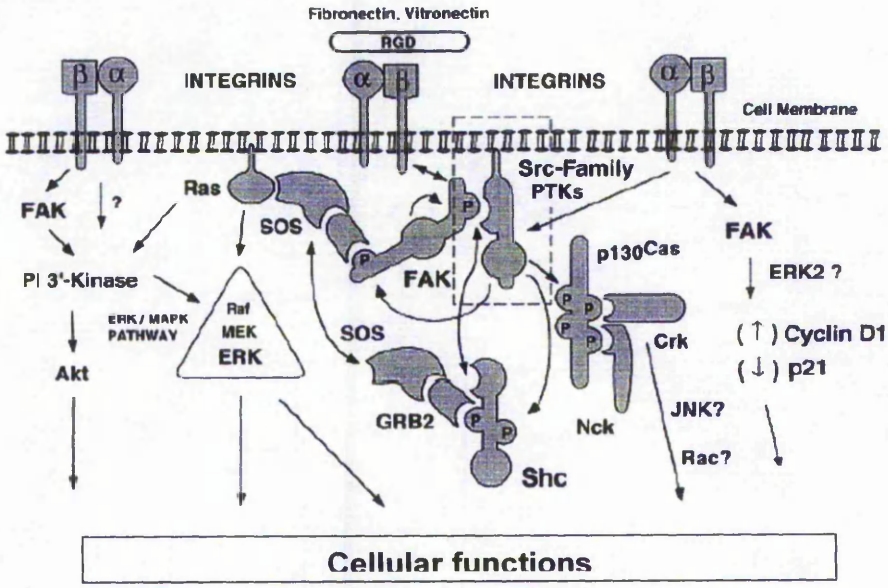


Figure 5.1.1. Integrin-stimulated signalling events involving FAK. FAK promotes the organised assembly of signalling complexes downstream of integrin receptor engagement with a variety of different ligands. FAK is predominantly implicated in the activation of downstream targets such as the ERK and JNK/mitogen-activated protein kinase cascades [adapted from Schlaepfer *et al.*, 1999].

The focus of this chapter is to characterise the cellular processes initiated and instigated during culture of the HOB and HFDF cells on the TG-modified collagen. Initially, this will involve identifying and determining the specific integrin receptors involved and characterising the associated cell signalling. Further downstream the transcription and translation levels of specific proteins will be identified and, ultimately, these will be related to cell behaviour.

5.2. RESULTS

5.2.1. Inhibition of integrin-mediated (RGD) binding

A feature of integrin-collagen binding appears to be the reliance on the triple helical structure of the collagen with the α1β1 and α2β1 integrins [Ruggiero *et al.,* 1994; Kern *et al.,* 1993; Tuckwell *et al.*, 1994; Vandenberg *et al.,* 1991]. Additionally, gelatin (denatured collagen type I without the 3-D structure) still maintains the inherent amino acid sequences of native triple-helical collagen (i.e. RGD and DGER) which are required to uphold cell growth and survival [Davis *et al.,* 2002]. As such, the provisional aim of this chapter is to identify and determine the extent of these binding capabilities in relation to the specific integrins involved. To determine the extent of integrin specific RGD-mediated binding of HOB cells to native and TG-treated collagen matrices, cell adhesion was inhibited using the synthetic peptide, GRGDTP and the control GRADSP peptide. The optimum concentration of the peptides used was shown to be 250μg/ml (used throughout in consecutive experiments) and was determined from a serial-dilution attachment inhibition assay (Figure 5.2.1A). Furthermore, it can be seen that a dose-dependent response exists for HOB cells when treated with the GRGDTP peptide- with 100% complete inhibition of cells attaching to native collagen and >60% inhibition for the tTG- and mTG-treated matrices when treated with 500μg/ml peptide concentration ($p<0.05$). It can be elucidated that this inhibition is as a direct consequence of the GRGDTP peptide as documented by Figure 5.2.1B which compares the inhibition of the HOB cells when treated with both the target peptide and the negative control peptide. Moreover, the TG-treated collagen matrices allowed more HOB cells to attach compared to the corresponding untreated collagen substrate suggesting that either more RGD-sites are available on the TG-treated matrices or, that the cells are exploiting alternative binding sites via upregulation or other compensatory mediated binding mechanisms. Further confirmation can be seen in Figure 5.2.1C which demonstrates a small but significant increase ($p<0.05$) in the HOB attachment characteristics when cultured on the TG-modified substrates.

5.2.2. Inhibition of α1 integrin-mediated binding

To determine the extent of α1 integrin-mediated binding of HOB cells to native and TG-treated collagen matrices, samples were inhibited using a function-

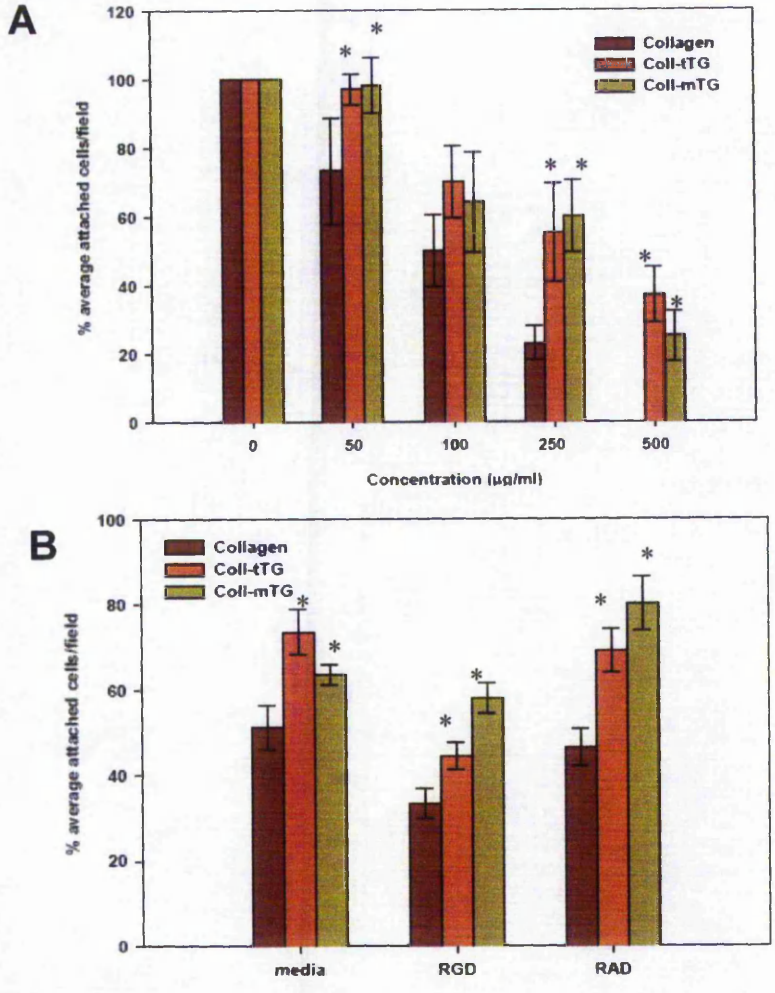


Figure 5.2.1. Inhibition of RGD integrin-mediated binding using synthetic peptide inhibitors. HOB cells ($2x10^5$ cells/ml) were incubated in a humidified-atmosphere incubator, at 37°C and with 5% CO_2, with the corresponding synthetic peptides for 15 minutes before being plated out on to the relevant substrates; native collagen, collagen-treated with 100µg/ml tTG or, collagen-treated with 100µg/ml mTG (enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg) as described in the Methods. Following 3h of culture, samples were fixed and subjected to the attachment analysis as described in Methods. (**A**) dose-dependent determination response of cells to GRGDTP peptide. Attachment is expressed as the percentage of the total number of cells attached for the non-treated sample. (**B**) response of cells to 250µg/ml of GRGDTP and GRADSP peptides. Attachment is expressed as the percentage of the total number of cells attached following 6h of culture on the non-treated collagen (ranging 160-300 cells/field). Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments. Results obtained following treatment with TG were compared to native collagen and values corresponding to $p<0.05$ when compared with the control are represented with a *.

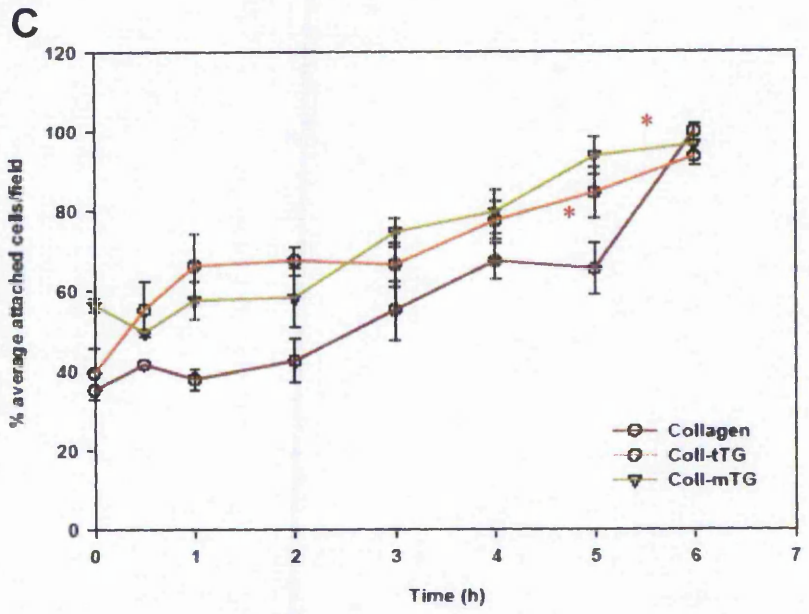


Figure 5.2.1. Inhibition of RGD integrin-mediated binding using synthetic peptide inhibitors. HOB cells ($2x10^5$ cells/ml) were incubated in a humidified-atmosphere incubator, at 37°C and with 5% CO_2, with the corresponding synthetic peptides for 15 minutes before being plated out on to the relevant substrates; native collagen, collagen-treated with 100µg/ml tTG or, collagen-treated with 100µg/ml mTG (enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg) as described in the Methods. Following 3h of culture, samples were fixed and subjected to the attachment analysis as described in Methods. (**C**) time-course response of HOB cells to 250µg/ml GRGDTP peptide. Attachment is expressed as the percentage of the total number of cells attached following 6h of culture on the non-treated collagen (ranging 160-300 cells/field). Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments with cell numbers ranging from 160-300 cells/field. A total of 5 random fields were selected. Results obtained following treatment with TG were compared to native collagen and values corresponding to $p<0.05$ when compared with the control are represented with a *. Line/data represented by a (red) coloured * indicate $p<0.05$ at all time points.

blocking antibody specific for α1. It can be seen that a dose-dependent response exists for the HOB cells when treated with the antibody with maximum inhibition achieved for all the substrates at the 1:1000 dilution value. However, consecutive experiments used a 1:2500 dilution factor (for the ease of comparison) which achieved an 80% inhibition of HOB cell attachment to native collagen and >60% for tTG- and mTG-treated matrices, respectively (Figure 5.2.2A). During the monitoring of the inhibition of the short-term attachment of HOB cells to the substrates (Figure 5.2.2B), it can be seen that, generally, a significantly higher ($p<0.05$) number of cells attach to the TG-treated collagen matrices compared to the native collagen at the corresponding time-points. Furthermore, negligible cells attach during the 1h of culture on the untreated collagen matrices and in the presence of the anti-α1 integrin antibodies but, in contrast, >15% cells attach on to both of the TG-modified collagen matrices. It can be seen that HOB cells inhibited with the anti-α1 integrin antibodies and cultured on native collagen never achieve greater than 20% attachment during the 6h of culture. In contrast, HOB cell attachment reached ~60% and ~45%, for the same time-point, when cultured on tTG- and mTG-treated matrices, respectively ($p<0.05$). Overall, a greater number of HOB cells attached to the tTG-modified collagen substrate and, also, at a faster rate compared to the other substrates.

5.2.3. Inhibition of α2 integrin-mediated binding

To determine the extent of α2 integrin-mediated binding of HOB cells to native and TG-treated collagen matrices, samples were inhibited using a function-blocking antibody specific for α2. It can be seen that a dose-dependent response exists for the HOB cells when treated with the antibody. However, even at a dilution factor of 1:500, complete inhibition of the TG-treated collagen matrices could not be achieved. Interestingly, complete inhibition was achieved on the attachment of the HOB cells cultured on native collagen at a 1:2500 dilution with a corresponding ~80% and ~60% attachment inhibition with the tTG- and mTG-modified matrices, respectively (Figure 5.2.3A). During the monitoring of the inhibition of the short-term attachment of HOB cells to the substrates (Figure 5.2.3B), it can be seen that a significantly greater ($p<0.05$) number of cells attach to the TG-treated collagen matrices compared to the native collagen at the corresponding time-points. Furthermore, negligible cells attach during the first 2h of culture on the untreated collagen

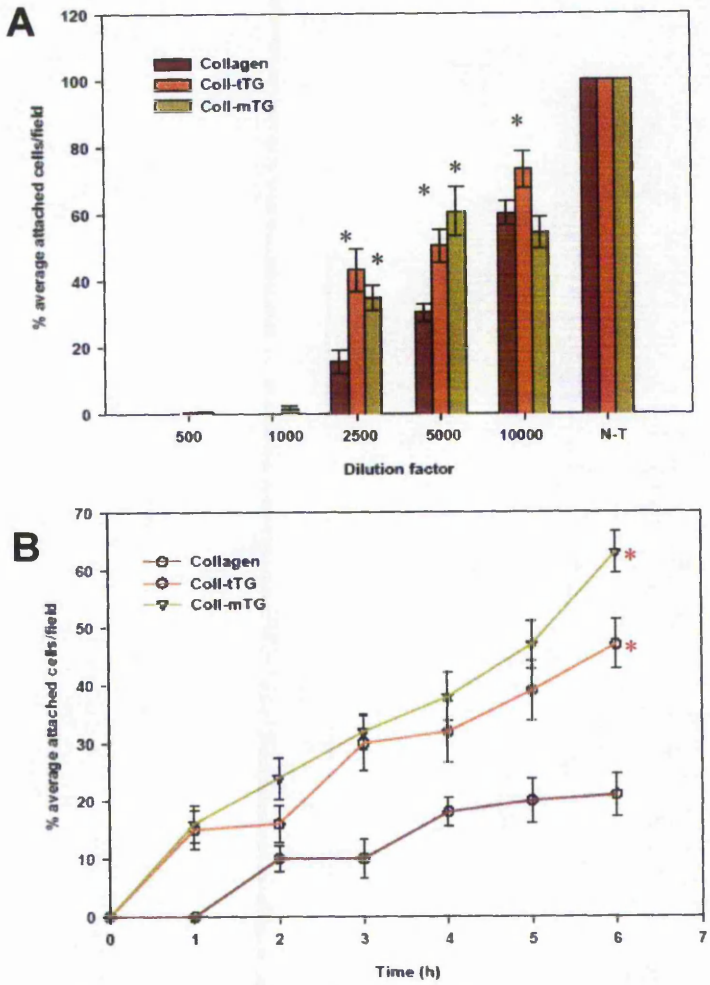


Figure 5.2.2. Inhibition of α1 integrin using a function-blocking antibody. HOB cells ($2x10^5$ cells/ml) were incubated in a humidified-atmosphere incubator, at 37°C and 5% CO_2, with the corresponding antibody for 15 minutes before being plated out on to the relevant substrates; native collagen, collagen-treated with 100µg/ml tTG or, collagen-treated with 100µg/ml mTG (enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg) as described in the Methods. Following 3h of culture, samples were fixed and subjected to the attachment analysis as described in Methods. (**A**) dose-dependent determination response of cells to the anti-α1 integrin antibody. (**B**) time-course response of HOB cells to 2500-fold dilution anti-α1 integrin antibody. Attachment is expressed as the percentage of the total number of cells attached following 6h of culture on the non-crosslinked collagen (in the absence of the anti-α1 integrin antibody). Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments with cell numbers ranging from 160-300 cells/field. A total of 5 random fields were selected. Results obtained following treatment with TG were compared to native collagen and values corresponding to $p<0.05$ when compared with the control are represented with a *. Line/data represented by a (red) coloured * indicate $p<0.05$ at all time points.

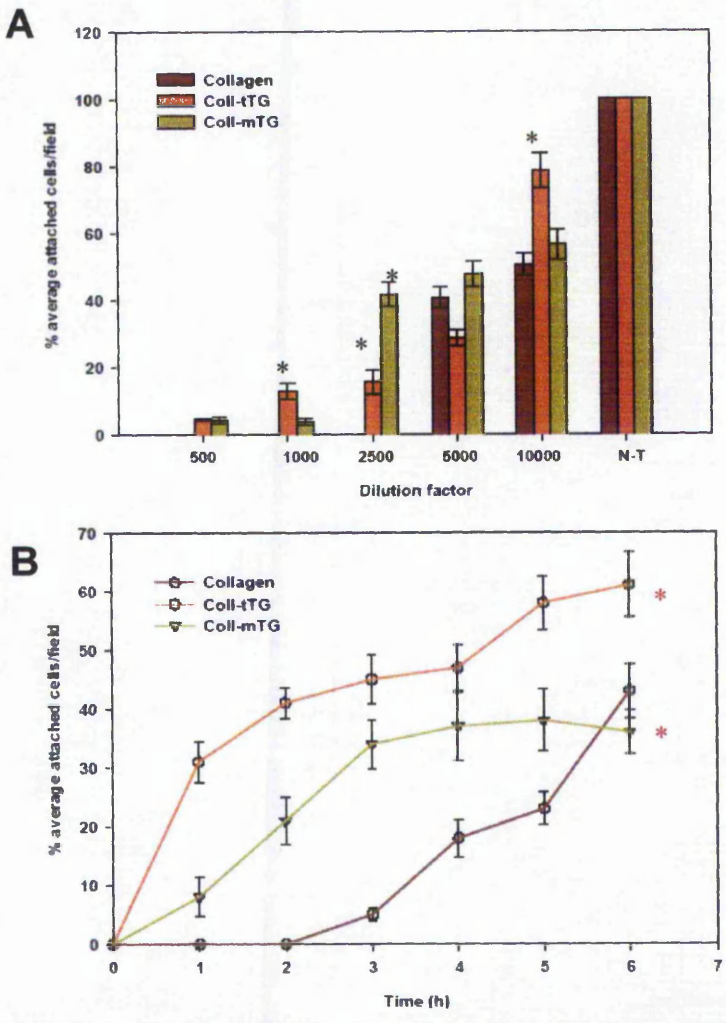


Figure 5.2.3. Inhibition of α2 integrin using a function-blocking antibody. HOB cells ($2x10^5$ cells/ml) were incubated in a humidified-atmosphere incubator, at 37°C and with 5% CO_2, with the corresponding antibody for 15 minutes before being plated out on to the relevant substrates; native collagen, collagen-treated with 100µg/ml tTG or, collagen-treated with 100µg/ml mTG (enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg) as described in the Methods. Following 3h of culture, samples were fixed and subjected to the attachment analysis as described in Methods. (**A**) dose-dependent determination response of cells to the anti-α2 integrin antibody. (**B**) time-course response of HOB cells to 2500-fold dilution anti-α2 integrin antibody. Attachment is expressed as the percentage of the total number of cells attached following 6h of culture on the non-crosslinked collagen (in the absence of the anti-α2 integrin antibody). Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments with cell numbers ranging from 160-300 cells/field. A total of 5 random fields were selected. Results obtained following treatment with TG were compared to native collagen and values corresponding to $p<0.05$ when compared with the control are represented with a *. Line/data represented by a (red) coloured * indicate $p<0.05$ at all time points.

matrices and in the presence of the anti-α2 integrin antibody but, in contrast, at the corresponding time-points, >40% and >15% cells attach to the tTG- and mTG-modified collagen matrices, respectively. It can be seen that, at all the time-points, a greater number of cells attached to the tTG-modified collagen and, also, at a faster rate than compared to the other substrates. Interestingly, at the 6h time-point, the equivalent number of HOB cells attached to the mTG-treated substrate is comparable with that of the native collagen matrix, at ~40% attachment, whereas for the tTG-modified collagen, over 60% of the cells become attached to the substrate.

5.2.5. Inhibition of β1 integrin-mediated binding

To determine the extent of β1 integrin-mediated binding of HOB cells to native and TG-treated collagen matrices, samples were inhibited using a function-blocking antibody specific for β1. From Figure 5.2.4A, it can be seen that a dose-dependent response exists for HOB cells cultured on the substrates: complete inhibition of the HOB cell attachment on native collagen substrate achieved at 1:5000 dilution, a 1:2500 dilution for the tTG-treated substrate and 1:1000 dilution for complete inhibition on the mTG-treated collagen matrix. Surprisingly, during the monitoring of the short-term attachment of the HOB cells on the matrices (Figure 5.2.4B), it can be seen that premature attachment occurred on the TG-treated matrices compared to the native collagen substrate. For the native collagen, HOB cell attachment only began following 3h of culture and reached ~35% after 6h. In contrast, HOB cells on the tTG-treated substrate began to attach only after 2h following culture and reached >40% after 6h. Interestingly, although the HOB cells on the mTG-modified matrix only began to attach after 4h of culture, a significantly greater number of cells attached at the 6h time-point (>60%) and the cell attachment occurred at a much quicker rate ($p<0.05$).

5.2.7. MMP and TIMP expression of HOB and HFDF cells cultured on native and TG-treated collagen matrices

The MMP and TIMP protein expression profiles of the HOB and HFDF cells, cultured on the native and TG-treated collagen matrices (3 hours) were determined using a multi-microarray assay, as described in the Methods and shown in Figures 5.2.5A and B, respectively. The densitometric analysis is

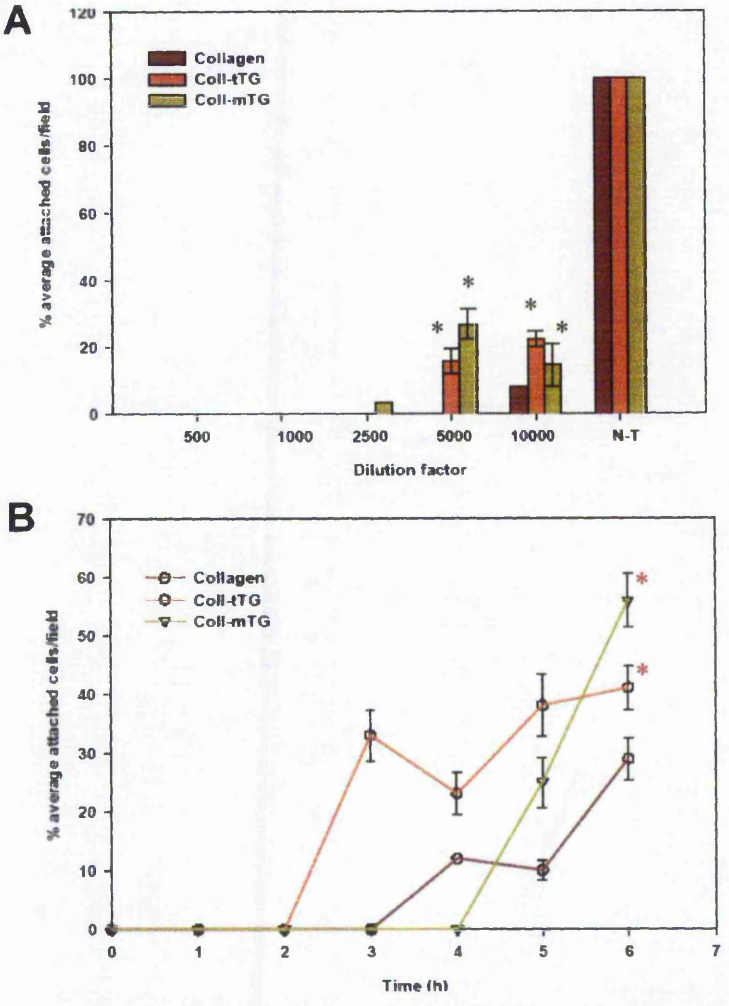
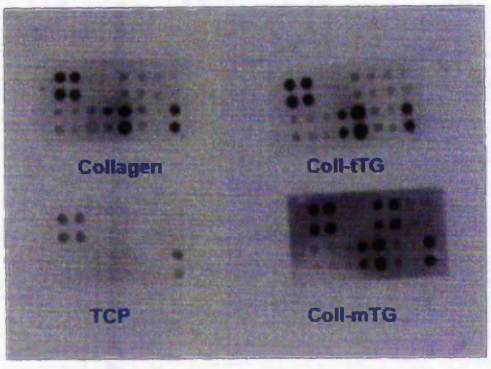
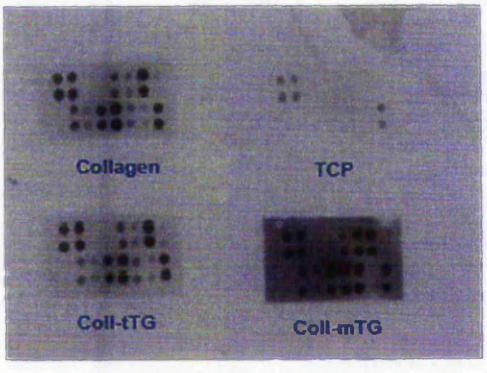


Figure 5.2.4. Inhibition of β1 integrin using a function-blocking antibody. HOB cells ($2x10^5$ cells/ml) were incubated in a humidified-atmosphere incubator, at 37°C and with 5% CO_2, with the corresponding antibody for 15 minutes before being plated out on to the relevant substrates; native collagen, collagen-treated with 100µg/ml tTG or, collagen-treated with 100µg/ml mTG (enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg) as described in the Methods. Following 3h of culture, samples were fixed and subjected to the attachment analysis as described in Methods. (**A**) dose-dependent determination response of cells to the anti-β1 integrin antibody. (**B**) time-course response of HOB cells to 2500-fold dilution anti-β1 integrin antibody. Attachment is expressed as the percentage of the total number of cells attached following 6h of culture on the non-crosslinked collagen (in the absence of the anti-β1 integrin antibody). Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from three independent experiments with cell numbers ranging from 160-300 cells/field. A total of 5 random fields were selected. Results obtained following treatment with TG were compared to native collagen and values corresponding to $p<0.05$ when compared with the control are represented with a *. Line/data represented by a (red) coloured * indicate $p<0.05$ at all time points.

A



B



C

	A	B	C	D	E	F	G	H
1	POS	POS	NEG	NEG	MMP-1	MMP-2	MMP-3	MMP-8
2	POS	POS	NEG	NEG	MMP-1	MMP-2	MMP-3	MMP-8
3	MMP-9	MMP-10	MMP-13	TIMP-1	TIMP-2	TIMP-3	TIMP-4	POS
4	MMP-9	MMP-10	MMP-13	TIMP-1	TIMP-2	TIMP-3	TIMP-4	POS

Figure 5.2.5. MMP and TIMP secretion profile of HOB and HFDF cells cultured on native and TG-treated collagen matrices. Micro-array analysis (RayBiotech, Insight Biotechnology Ltd., Middlesex, UK) for secreted MMPs and TIMPs of (**A**) HOB and (**B**) HFDF cells following 3h culture on native and 100µg/ml TG-treated collagen matrices (enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg). Samples were prepared and analysed as described in the Methods. (**C**) reference array layout supplied by RayBiotech corresponding to the micro-array assay. Results are from a single experiment of pooled triplicate well samples.

summarised in Table 5.2.1. In the case of the HOB cells, negligible amounts of MMPs were expressed by cells when cultured on tissue culture plastic (TCP); although trace amounts of TIMP-1 and TIMP-2 were found to be present. In contrast, HOB cells cultured for 3h on native collagen expressed a wide range of MMPs and TIMPs including MMP-1, -2, -3, -8, -9, -10 and -13 as well as TIMP-1, -2, -3 and -4. However, HOB cells cultured on tTG-treated collagen substrates demonstrated an altered profile which consisted of an increase of MMP-8, TIMP-1, TIMP-2 and TIMP-4 expression. Furthermore, cells cultured on the mTG-treated collagen substrates expressed increases of MMP-1, MMP-2 and TIMP-1 compared to the profile of the cells cultured on native collagen.

A similar profile can be noted for the HFDF cells as seen in Figure 5.2.5B. Interestingly, the cells cultured on TCP were shown to express trace amounts of MMP-1, MMP-2 and TIMP-3. In comparison, the full spectrum of MMP-1, -2, -3, -8, -9, -10 and -13 as well as TIMP-1, -2, -3 and -4 was expressed when the cells were cultured on a native collagen. In the case of HFDF cells cultured on the tTG-treated collagen, only MMP-8 expression was increased. In contrast, when the cells were grown on the mTG-treated collagen, increases in the expression of MMP-2, -3 and TIMP-1, -2 and -3 with a corresponding decrease in MMP-10 were detected.

5.2.10. Northern blotting for Coll-αI(I), MMP-1 and MMP-2 expression in HOB and HFDF cells cultured on native and TG-treated collagen matrices

To confirm and explain the MMP expression profiles identified the previous results, it was deemed necessary to explore the up- or down-regulation of Coll-αI(I), MMP-1 and MMP-2 RNA for the cell lines using Northern blotting. Equal loading of total RNA samples, derived from HOB and HFDF cells that were cultured on native and TG-treated collagen matrices (confirmed by agarose/formaldehyde denaturing gel in Figure 5.2.6) were probed for Coll-αI(I), MMP-1 and MMP-2 RNA expression as seen in Figure 5.2.7, Figure 5.2.8 and Figure 5.2.9, respectively. Images were analysed by Phoretix 1D image analysis (densitometry) software, normalised against the relevant cyclophilin loading and summarised in the corresponding histogram.

	HOB			HFDF		
	Collagen	Coll-tTG	Coll-mTG	Collagen	Coll-tTG	Coll-mTG
MMP-1	1.00	1.03	1.48	1.00	1.01	0.92
MMP-2	1.00	1.05	1.87	1.00	1.02	1.76
MMP-3	1.00	1.03	1.05	1.00	1.01	1.19
MMP-8	1.00	1.11	0.96	1.00	1.10	0.99
MMP-9	1.00	1.02	0.99	1.00	1.02	1.01
MMP-10	1.00	1.03	0.98	1.00	0.92	0.89
MMP-13	1.00	0.97	0.95	1.00	0.99	0.99
TIMP-1	1.00	1.23	1.19	1.00	1.01	1.11
TIMP-2	1.00	1.11	1.01	1.00	0.98	1.19
TIMP-3	1.00	1.02	1.04	1.00	1.06	1.83
TIMP-4	1.00	1.13	1.00	1.00	0.99	1.00

Table 5.2.1. Densitometric analyses of MMP and TIMP microarray secretion profiles of HOB and HFDF cells cultured on native and TG-treated collagen matrices. The micro-array images for the secreted MMPs and TIMPs of HOB and HFDF cells following 3h culture on native collagen, collagen-treated with 100µg/ml tTG or, collagen-treated with 100µg/ml mTG (enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg). Spots were analysed using ImageJ densitometry software (http://rsb.info.nih.gov/ij/index.html) and normalised against background values prior to comparison with native collagen control sample. Results are from a single experiment of pooled triplicate well samples.

It can be seen in Figure 5.2.7D that variations exist in the densitometric analysis of Coll-αI(I) expression between the cells cultured on native and TG-treated collagen. HFDF cells express a greater amount of Coll-αI(I) RNA when cultured on a native collagen substrate as opposed to when the cells are grown on either of the TG-treated matrices. As such, it can be seen that both the tTG- and mTG-treated matrices induced a similar Coll-αI(I) RNA expression profile. In the case of HOB cells cultured on these matrices, it can be noted that the expression levels of Coll-αI(I) by these cells were comparable when cultured on native collagen and during culture on the mTG-treated collagen. Unfortunately, no sample appeared to have been loaded for the tTG-treated collagen sample. The histogram presented by Figure 5.2.8D summarises the results for MMP-1 expression between cells cultured on native collagen and on the TG-treated collagen matrices. It can be seen that little variation exists for the HFDF samples when cultured on native and either of the TG-modified matrices. However, in contrast, a distinct difference can be noted for the osteoblastic cell line. The HOB cells expressed more MMP-1 RNA when cultured on the mTG-modified substrate; approximately a 3-fold increase compared to the cells when cultured on a native collagen substrate. A similar behaviour can be noted for the cells cultured on the tTG-treated substrate- although only a 2-fold increase was apparent compared to the native collagen sample.

Figure 5.2.9D documents the analyses of MMP-2 RNA expression for both the HFDF and HOB cells when cultured on native and TG-treated collagen matrices. It can be seen that both of the TG-treated collagen matrices induce higher levels of MMP-2 expression for HFDF cells during culture compared to growth only just native collagen. As such, ~35% and ~15% increases in the RNA levels are demonstrated by the tTG-treated and mTG-treated matrices, respectively. In contrast, a major change can be noted for the osteoblastic cell line. When the HOB cells were cultured on the same substrates, approximately a 300% and 450% increase in the MMP-2 RNA expression level occurred for the tTG-treated and mTG-treated matrices, respectively, compared to culture on the native collagen substrate.

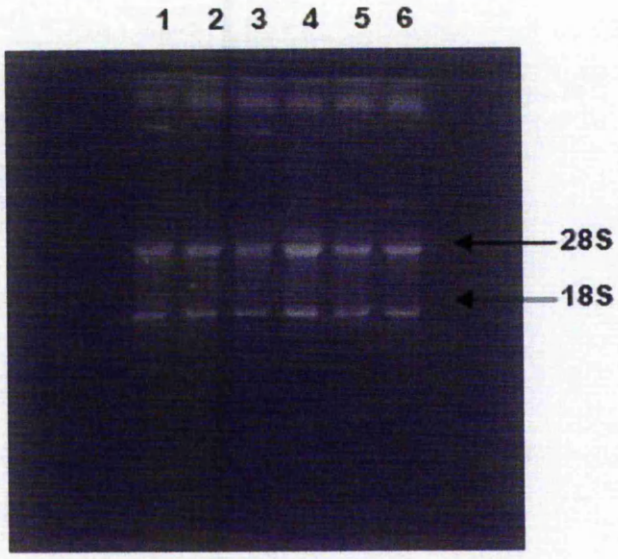


Figure 5.2.6. Agarose/formaldehyde gel electrophoresis of HOB and HFDF cells cultured on native and TG-treated collagen matrices. Total RNA of $2x10^6$ HOB and HFDF cells, cultured on native collagen, collagen-treated with 100µg/ml tTG or, collagen-treated with 100µg/ml mTG matrices (enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg) was extracted using TRI reagent before being resolved (20µg/lane) on a denaturing agarose/formaldehyde gel. Gels electrophoresis was performed at 80V for approximately 45 minutes. Arrows demonstrate intact 28S and 18S rRNA units. Lane 1, HFDF cells cultured on untreated (native) collagen; lane 2, HFDF cells cultured on tTG-treated collagen matrix; lane 3, HFDF cells cultured on mTG-treated collagen matrix; lane 4, HOB cells cultured on untreated (native) collagen; lane 5, HOB cells cultured on tTG-treated collagen matrix; lane 6, HOB cells cultured on mTG-treated collagen matrix. Results are from a single experiment of pooled triplicate samples.

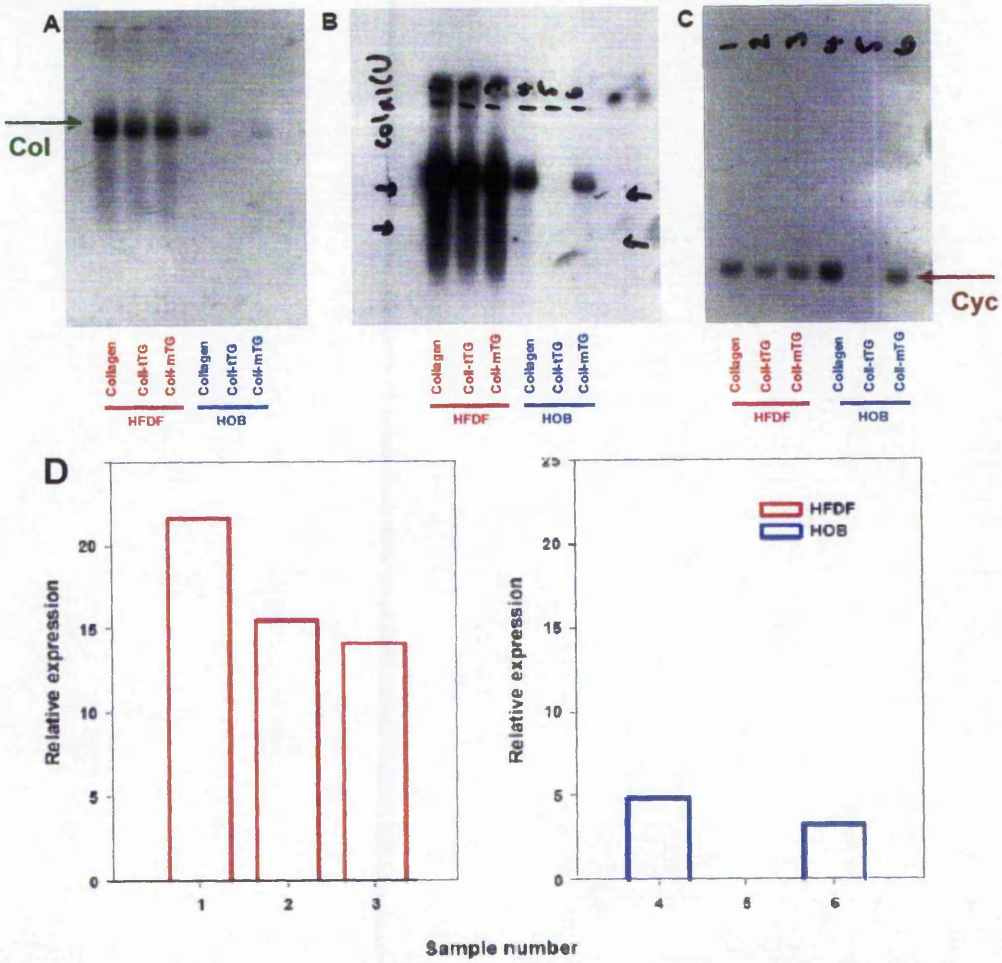


Figure 5.2.7. Collagen I expression in HFDF and HOB cells cultured on native and TG-treated collagen matrices. Total RNA (20µg/lane) of $2x10^6$ HOB and HFDF cells, cultured on native collagen, collagen-treated with 100µg/ml tTG or, collagen-treated with 100µg/ml mTG matrices (enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg) subjected to Northern transfer and hybridisation with collagen I cDNA probes as outlined in the Methods. Northern blot of (**A**) collagen I following 2h exposure, (**B**) collagen I following overnight exposure, (**C**) cyclophilin expression of the samples. (**D**) Values represent the corrected value for total RNA loading by normalisation against the cyclophilin membrane band density accordingly using Phoretix 1D image analysis software. Lane 1, HFDF cells cultured on untreated (native) collagen; lane 2, HFDF cells cultured on tTG-treated collagen matrix; lane 3, HFDF cells cultured on mTG-treated collagen matrix; lane 4, HOB cells cultured on untreated (native) collagen; lane 5, HOB cells cultured on tTG-treated collagen matrix; lane 6, HOB cells cultured on mTG-treated collagen matrix. Cyc corresponds to cyclophilin. Results are from a single experiment of pooled triplicate samples.

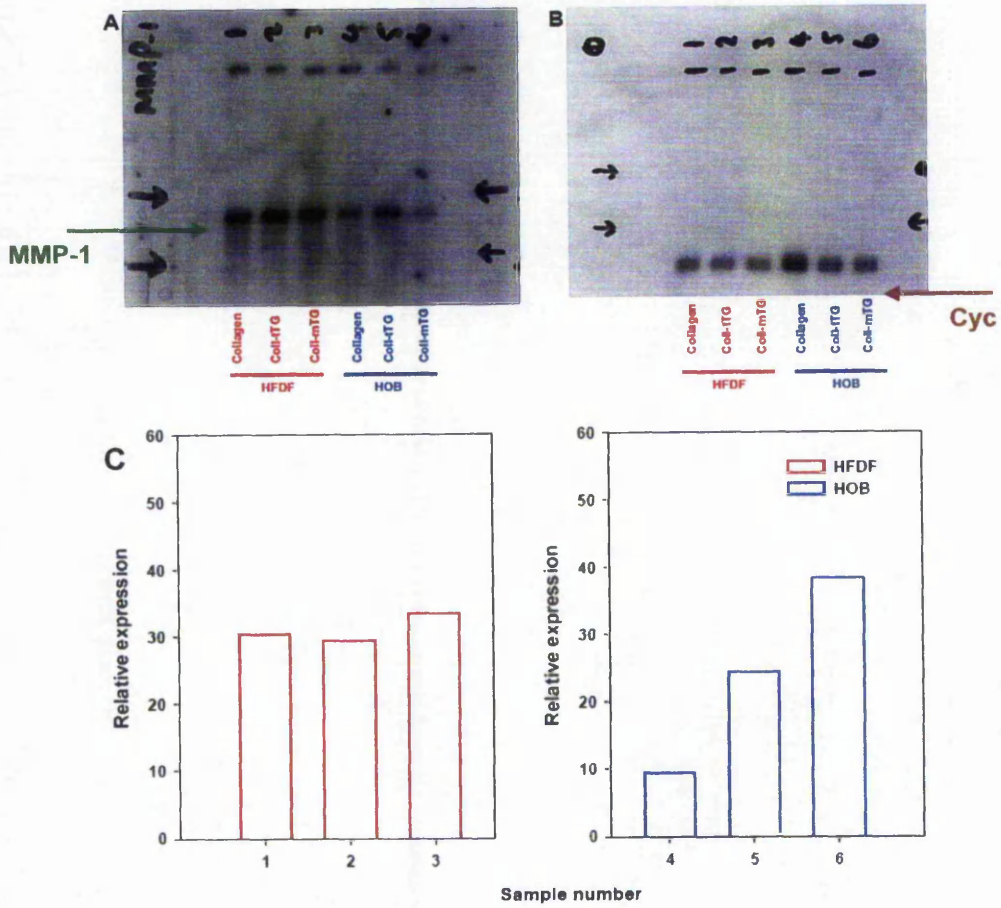


Figure 5.2.8. MMP-1 expression in HFDF and HOB cells cultured on native and TG-treated collagen matrices. Total RNA (20µg/lane) of $2x10^6$ HOB and HFDF cells, cultured on native collagen, collagen-treated with 100µg/ml tTG or, collagen-treated with 100µg/ml mTG matrices (enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg) subjected to Northern transfer and hybridisation with MMP-1 cDNA probes as outlined in the Methods. Northern blot of (**A**) MMP-1 following 4h exposure (**B**) cyclophilin expression of the samples. (**C**) Values represent the corrected value for total RNA loading by normalisation against the cyclophilin membrane band density accordingly using Phoretix 1D image analysis software. Lane 1, HFDF cells cultured on untreated (native) collagen; lane 2, HFDF cells cultured on tTG-treated collagen matrix; lane 3, HFDF cells cultured on mTG-treated collagen matrix; lane 4, HOB cells cultured on untreated (native) collagen; lane 5, HOB cells cultured on tTG-treated collagen matrix; lane 6, HOB cells cultured on mTG-treated collagen matrix. Results are from a single experiment of pooled triplicate samples. Cyc corresponds to cyclophilin. Results are from a single experiment of pooled triplicate samples.

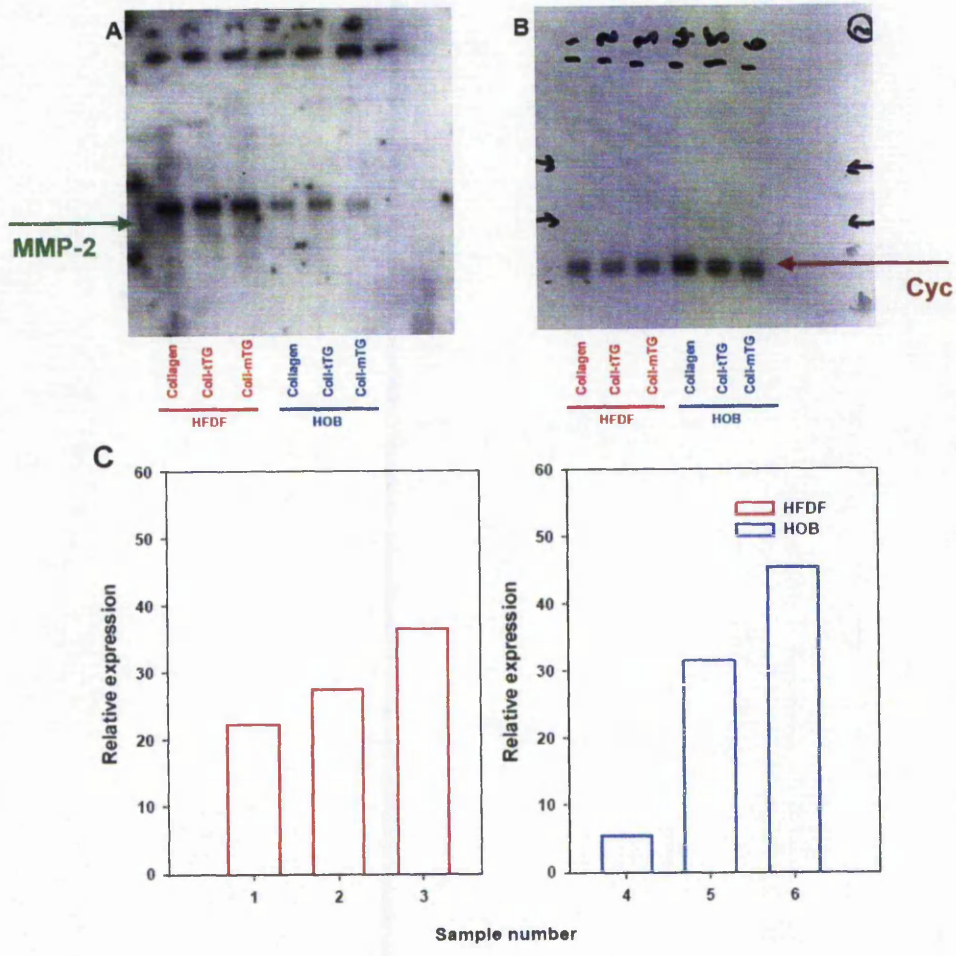


Figure 5.2.9. MMP-2 expression in HFDF and HOB cells cultured on native and TG-treated collagen matrices. Total RNA (20µg/lane) of $2x10^6$ HOB and HFDF cells, cultured on native collagen, collagen-treated with 100µg/ml tTG or, collagen-treated with 100µg/ml mTG matrices (enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg) subjected to Northern transfer and hybridisation with MMP-2 cDNA probes as outlined in the Methods. Northern blot of (**A**) MMP-2 following 15h exposure (**B**) cyclophilin expression of the samples. (**C**) Values represent the corrected value for total RNA loading by normalisation against the cyclophilin membrane band density accordingly using Phoretix 1D image analysis software. Lane 1, HFDF cells cultured on untreated (native) collagen; lane 2, HFDF cells cultured on tTG-treated collagen matrix; lane 3, HFDF cells cultured on mTG-treated collagen matrix; lane 4, HOB cells cultured on untreated (native) collagen; lane 5, HOB cells cultured on tTG-treated collagen matrix; lane 6, HOB cells cultured on mTG-treated collagen matrix. Cyc corresponds to cyclophilin. Results are from a single experiment of pooled triplicate samples.

5.2.11. FAK-Y397 phosphorylation of HOB cells cultured on native and TG-treated collagen matrices

In order to identify any variations to cell signalling pathways that may have been induced by the culture on different substrates, the phosphorylation of FAK-Y397 was monitored during a 6h culture period on native and the TG-treated collagen matrices and shown in Figure 5.2.10A. The histogram (Figure 5.2.10B) summarises the densitometric results for the Western blotting of phosphorylated FAK-Y397 for HOB cells, normalised against tubulin-α loading, at the 1h and 6h time-points. It can be seen that during the first hour of culture, FAK-Y397 phosphorylation is higher on both of the TG-treated collagen matrices: a 2-fold and 3-fold increase, respectively, compared to that of the cells cultured on just native collagen. Interestingly, at the 6h time-point, phosphorylation of FAK-Y397 remains relatively constant for the cells cultured on native collagen. However, in contrast, for both of the TG-treated matrices, FAK-Y397 phosphorylation significantly increases 4-fold compared to the native collagen at the same time-point.

Figures 5.2.11A, B and C characterise FAK-Y397 phosphorylation of the HOB cells, normalised against tubulin-α loading, via Western blotting, during a long-term (1-24h) culture period on native and the TG-treated collagen matrices. It can be seen that although a time-dependent phosphorylation occurs for all three substrates, the TG-crosslinked matrices induced greater FAK-Y397 phosphorylation compared to culture on native collagen. Interestingly, a decrease in the level of phosphorylation of FAK-Y397 was detected at the 6h time-point during culture on native and the tTG-treated collagen substrates but did not occur during culture on the mTG-treated matrix. It can be noted that the greatest FAK-Y397 phosphorylation occurred with the mTG-treated collagen substrate- approximately 15-20% increase at all corresponding time-points. The histogram presented in Figure 5.2.12 shows the normalised values of phosphorylated FAK-Y397, relative to each matrix and time-point, and indicates significant increases ($p<0.05$) of FAK-Y397 phosphorylation during culture on the TG-treated collagen matrices. The greatest amount of phosphorylated FAK-Y397 was found to occur at the 6h time-point with the mTG-treated collagen matrix.

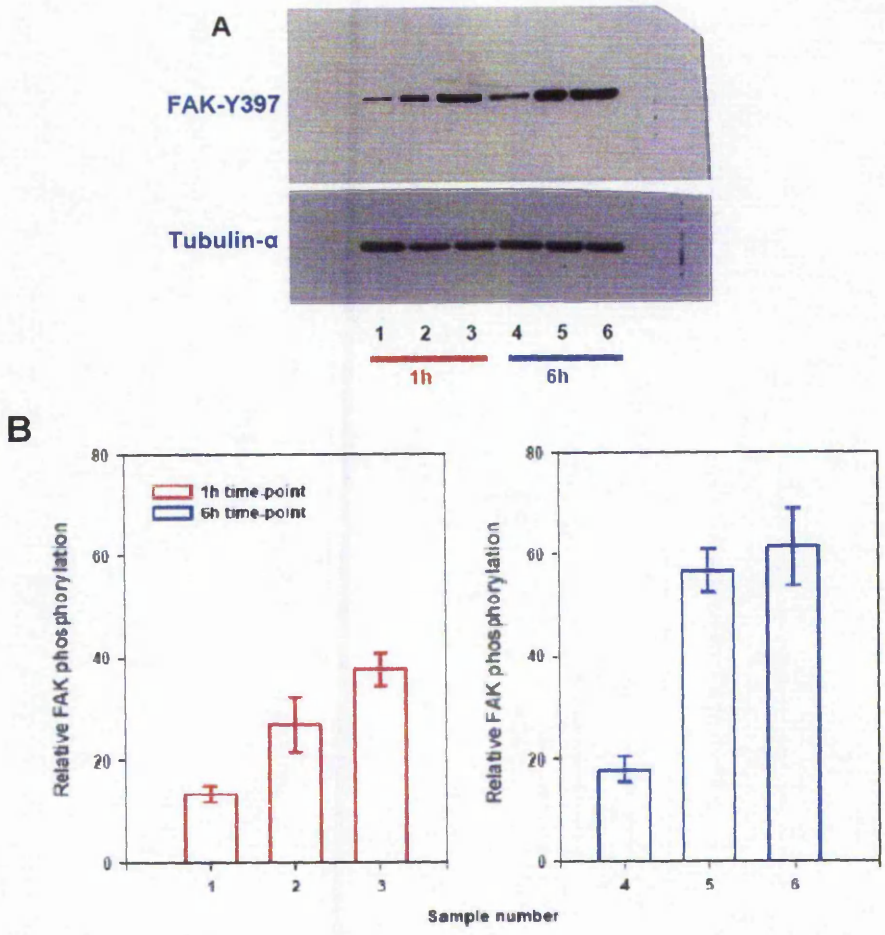


Figure 5.2.10. FAK-Y397 phosphorylation of HOB cells cultured on native and TG-treated collagen matrices. $2x10^6$ HOB cells, cultured on native collagen, collagen-treated with 100µg/ml tTG or, collagen-treated with 100µg/ml mTG matrices (enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg), under low serum conditions (2%v/v), were lysed and probed for FAK-Y397 phosphorylation and tubulin expression at the 1h and 6h time-points during culture using a rabbit polyclonal anti-FAK-Y397 antibody (Upstate, Buckingham, UK; at 1:1000 dilution) and mouse MAb anti-tubulin-α antibody (Santa Cruz Biotechnology, Calne, UK; at 1:1000 dilution), respectively, as described in the Methods. (**A**). Western blots of samples: lane 1, HOB cells cultured on untreated (native) collagen (1h); lane 2, HOB cells cultured on tTG-treated collagen matrix (1h); lane 3, HOB cells cultured on mTG-treated collagen matrix (1h); lane 4, HOB cells cultured on untreated (native) collagen (6h); lane 5, HOB cells cultured on tTG-treated collagen matrix (6h); lane 6, HOB cells cultured on mTG-treated collagen matrix (6h). (**B**) Values represent the corrected value for FAK-Y397 phosphorylation by normalisation against the tubulin band density using ImageJ analysis software. Results are from two independent experiments.

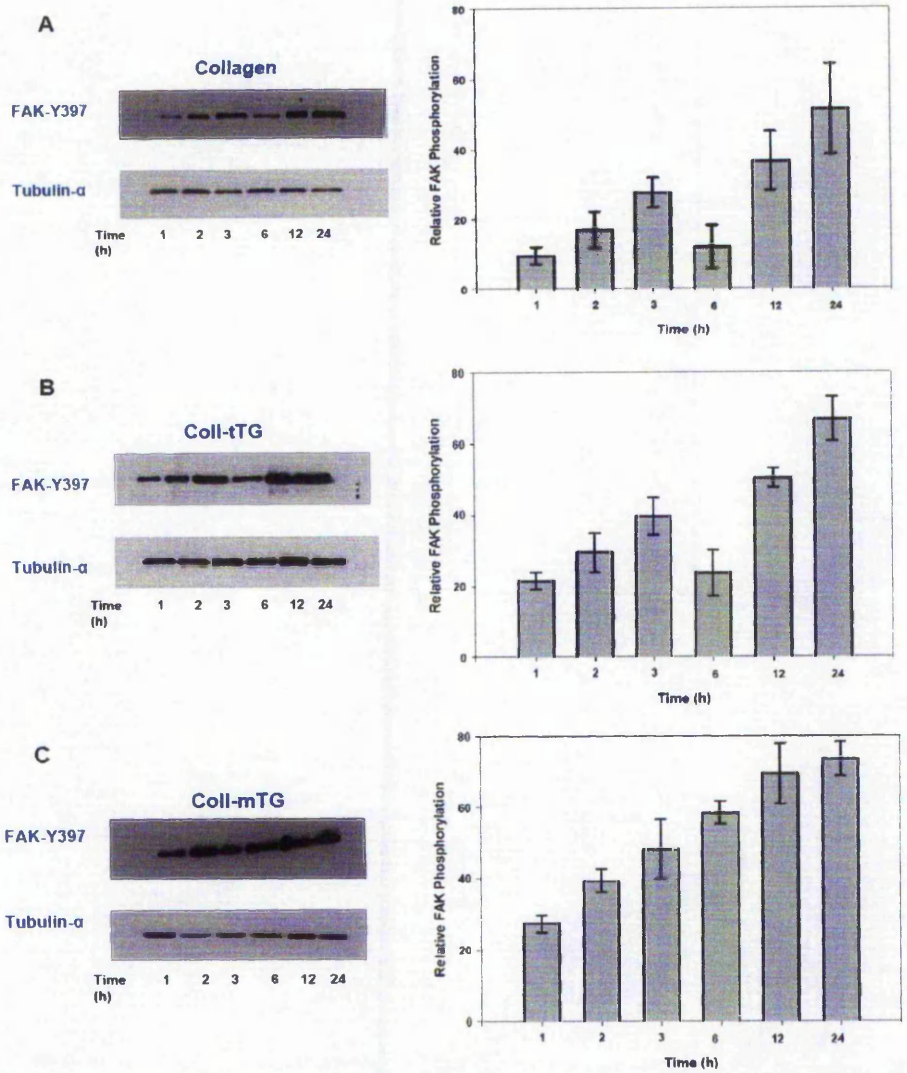


Figure 5.2.11. Long-term FAK-Y397 phosphorylation for HOB cells cultured on native and TG-treated collagen matrices. $2x10^6$ HOB cells, cultured on (**A**) native collagen; (**B**) collagen-treated with 100μg/ml tTG or (**C**) collagen-treated with 100μg/ml mTG matrices (enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg), under low serum conditions (2%v/v), for 24h were lysed and probed for FAK-Y397 phosphorylation and tubulin expression using a rabbit polyclonal anti-FAK-Y397 antibody (Upstate, Buckingham, UK; at 1:1000 dilution) and mouse MAb anti-tubulin-α antibody (Santa Cruz Biotechnology, Calne, UK; at 1:1000 dilution), respectively, as described in the Methods.. Densitometric quantification of blots was performed using ImageJ analysis software. Values represent the corrected value for FAK-Y397 phosphorylation by normalisation against the tubulin band density accordingly. Lane 1, HOB cells cultured on appropriate matrix for 1h; lane 2, HOB cells cultured on appropriate matrix for 2h; lane 3, HOB cells cultured on appropriate matrix for 3h; lane 4, HOB cells cultured on appropriate matrix for 6h; lane 5, HOB cells cultured on appropriate matrix for 12h; lane 6, HOB cells cultured on appropriate matrix for 24h. Results are from two independent experiments.

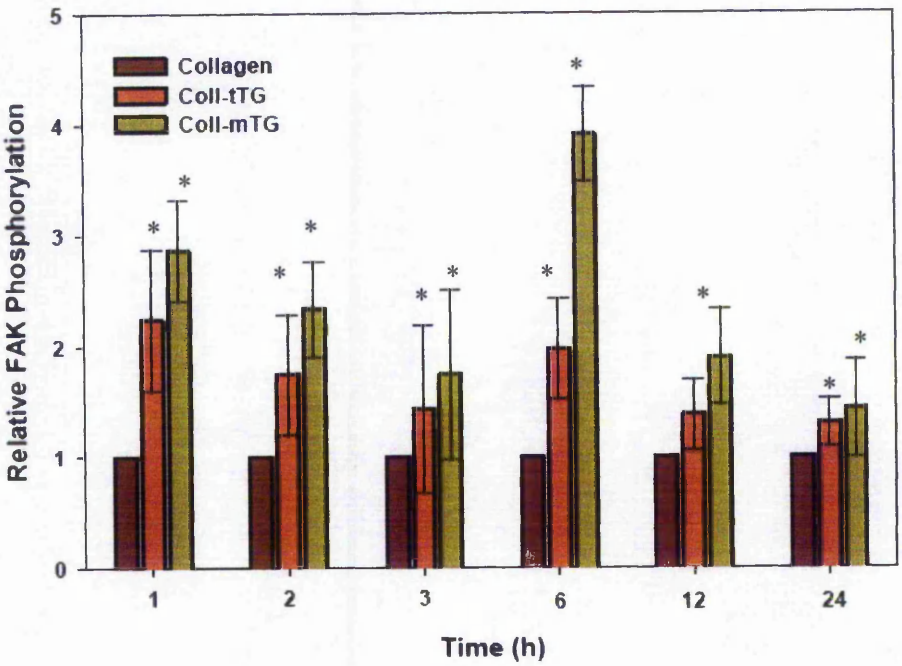


Figure 5.2.12. FAK-Y397 phosphorylation for HOB cells cultured on native and TG-treated collagen matrices. $2x10^6$ HOB cells, cultured on (**A**) native collagen; (**B**) collagen-treated with 100µg/ml tTG or (**C**) collagen-treated with 100µg/ml mTG matrices (enzyme activities: tTG: 11500-13000 Units/mg; mTG: 16000-25000 Units/mg), under low serum conditions (2%v/v), for 24h were lysed and probed for FAK-Y397 phosphorylation and tubulin expression using a rabbit polyclonal anti-FAK-Y397 antibody (Upstate, Buckingham, UK; at 1:1000 dilution) and mouse MAb anti-tubulin-α antibody (Santa Cruz Biotechnology, Calne, UK; at 1:1000 dilution), respectively. Values represent the FAK-Y397 phosphorylation of HOB cells on the TG-treated collagen matrices (normalised against specific tubulin intensity) relative to the FAK-Y397 phosphorylation during culture on native collagen. Densitometry analysis was performed using ImageJ analysis software as described in the Methods. Statistical analysis was carried out using the one-way ANOVA test and results are the mean values ±SD from two independent experiments. Results obtained following treatment with TG were compared to native collagen and values corresponding to $p<0.05$ when compared with the control are represented with a *.

5.3. DISCUSSION

In this study, the effect of cell attachment on native and TG-treated collagen matrices following inhibition of specific integrins with functional blocking antibodies was investigated and, in agreement with the literature, demonstrated that inhibition of the α1, α2 and β1 integrins reduced cell adhesion to native collagen. Control experiments using low serum media as well as preincubating cells with the antibodies eliminated the possibility of the presence of supplementary growth/adhesion factors and new synthesis or cell division, respectively. On culturing the cells on the TG-modified matrices, it can be seen that a greater number of cells attach to the modified substrates- that is to say that cell adhesion is not inhibited by the antibodies compared to that on native collagen. This observation can be partly explained by the hypothesis proposed in the previous chapter- that the exposure of new cryptic attachment sites may have become available following the TG-mediated modification. However, this exposure of new cryptic sites, itself, may not be sufficient for the apparent increase in cell attachment- as the specific integrin subunits themselves are targeted by the antibodies. Thus, alternative explanations such as the possible exposure of non-integrin binding sites following the TG-modification or, the presence of a greater number of binding sites (inferred from the antibody inhibition assays) seem plausible and may also clarify the observed cell behaviour. Furthermore, as it was previously proposed that the TG-modified collagens may possibly possess a dual fibrillar-gelatinous structure (i.e. cellular recognition), this physical characteristic in itself may also contribute to the observed cell adhesion behaviour by virtue of its affinity to fibrillar or non-fibrillar collagen: causing the corresponding up- or down-regulation of certain integrins or, even modifying the preference of other integrins to the new substrate. However, it may also be possible that a simple case of a "compensatory effect" occurring whereby the cells utilise alternative binding mechanisms due to the blocking of the main integrin-mediating subunit.

A detailed MMP and TIMP expression profile of the HOB and HFDF cells can be seen in the microarray assays, summarised as Table 5.2.1, from which it can be noted that the additional upregulation of MMP-3 and the TIMPs become more apparent during culture on the TG-treated collagen matrices. It is known MMP-3 (stromelysin 1) is not only capable of degrading the different types of collagens and proteoglycans but is also associated with the activation

of other MMPs such as MMP-2 and MMP-9 [Zeisel *et al.,* 2004; Saus *et al.,* 1988]. The corresponding TIMPs profile further highlights the concomitant regulation of the MMP expression: as it is known that each TIMP binds with both a distinct rate of interaction and affinity to a specific target MMP- usually in 1:1 or 2:2 stoichiometrical manner [Palosaari, 2003]. Thus, it may be postulated that this expression profile is not only tightly regulated but may also affect and alter the integrin-mediated downstream cellular signalling and processing of the cells. It is likely that the cells sense and treat the TG-modified matrices to be more gelatin-like than if it were native triple-helical collagen and, hence, expresses the relevant (and up-regulated) MMPs to aid its apparent matrix remodelling and cellular migration. Thus, the critical parameter relates the ratio of the MMPs to TIMPs in order to define whether the enzymes become proteolytically active. As such, the concomitant release of the TIMPs may be an additional cellular response to prevent "excess" degradation by the cells or, more likely, that their expression is required due to the inability of the MMPs to digest the (assumed) substrate and, as an in-built safety mechanism, causes the supplementary expression of TIMPs to prevent self-degradation. Additionally, the gelatinases have been known to form complexes with the TIMPs when the enzymes are in the latent form. The complex of latent MMP-2 (pro-MMP-2) with TIMP-2 serves to facilitate the activation of pro-MMP-2 at the cell surface and may form the basis of a tightly regulated cellular process [Bigg *et al.,* 2001; Hernandez-Barrantes *et al.*, 2001; Kinoshita *et al.,* 1998]. These observations suggest that either the collagen has been modified beyond recognition for the cells or, that the modified matrix is preferred to more than native collagen for the HOB remodelling purposes. In either case, the initial identification step of the ECM by the cells is mediated by the integrins and hence, any deviation would result in a change in the downstream signalling pathways.

When HOB cells are cultured on the TG-treated collagen matrices, it can be seen that not only do greater levels of phosphorylated FAK-Y397 become more apparent but the phosphorylation of the FAK-Y397 occurs at a much earlier time compared to culture on native collagen. As such, the relative increase of FAK-Y397 phosphorylation suggest that either a specific signalling pathway may be enhanced or, in contrast, the simultaneous activation of multiple pathways may be induced in response to the cell-ECM interaction between the cell and the presence of a more biocompatible/suitable substrate

for the cells. However, it may be suggested that the detected increase in phosphorylated FAK-Y397 levels may be attributed to an increase in cell number or due to the presence of serum co-factors. However, the use of low serum containing medium and the limited time-course of the assay minimised such a possibility. However, care must also be taken prior to drawing any definitive conclusions, as not only were the total FAK level not assayed but, also, FAK activation can occur via other alternative and multiple phosphorylation sites- occurring at six or more possible sites [Schlaeper and Hunter, 1998]. Furthermore, although the results were from two independent experiments, this low sample number may be subject to variability itself. Interestingly, it has been reported that fibroblast FAK-Y397 is phosphorylated and activated in response to contact with both 2-D and 3-D collagen matrices [Rockel and Krieg, 1994] and, to that effect, we have demonstrated such a behaviour with the HFDF cells on native and TG-treated collagen substrates during this investigation.

Although the primary activation and regulation of FAK occurs via its integrin engagement, it has also been shown that its regulation may also be, in part, attributed to matrix-derived mechanical stimuli: an observation documented by its yet undefined role as a mechanoreceptor [Xia *et al.,* 2004]. As such, as it has been demonstrated that the physical/mechanical properties of the collagen matrices are changed following TG-treatment, it may be postulated that these mechanical properties of the modified matrices may also be able to elicit or enhance a FAK-Y397 phosphorylating pathway. A similar observation was noted by Wang and colleagues who showed that the rigidity of collagen gels controls the expression of focal adhesion complex proteins which are, themselves, mediated by the α2β1 integrin [Wang *et al.,* 2003]. To further emphasise the role of FAK as a central mediator of multiple signalling pathways, it has been reported that osteopontin (OPN) may trigger alkaline phosphatase gene expression via integrin-mediated FAK activation [Liu *et al,* 1997]. Moreover, the same authors indicate that OPN is not only responsible for early differentiation but also confirm the different roles of OPN and collagen expression in osteoblast differentiation; the former protein acting as a trigger whilst the latter induces spontaneous induction [Liu *et al.,* 1997]. As it seems, our results further confirm such a finding- although it may be stressed that gene expression may not translate or correspond to overall protein secretion at the reported time-scale. Even so, we have demonstrated that an increase in

the expression of OPN and ALP occurs during culture of HOB cells on TG-treated collagen matrices- suggesting the earlier onset of osteoblast differentiation.

From the combination of zymographs and the integrin inhibition studies, we demonstrate that additional integrin-mediated binding occurs and leads to a corresponding increase in the expression of certain MMPs. Earlier research has also demonstrated that in addition to mediating lattice contraction, the α2β1 integrin regulates transcription of MMP-1 levels and this increase in mRNA levels correlates with an increase in the MMP-1 activity [Mauch *et al,* 1989]. However, contrastingly, the Northern blots demonstrate an increase in the RNA expression MMP-1 during cell culture on the modified matrices and that this relative increase does not appear to be translated to the actual amount of MMP-1 expressed by the cell nor to its enzymatic activity which can be inferred from the collagen zymographs. Furthermore, the blots also highlight the increase of collagen I synthesis as well as the over-expression of MMP-2 during culture on the TG-treated matrices. As such, this elevated level of gelatinase has been further confirmed by antibody-mediated micro-array data, gelatin zymographs and permutations of receptor-targeting inhibition assays. This contradictory point may simply be explained by the fact that the Northern blots are based on a single pooled sample. Hence, further experiments are required to define and determine the exact transcription and translation processes that occur following specific integrin-mediated cell-ECM interaction.

CHAPTER 6:
GENERAL DISCUSSION

CHAPTER 6: GENERAL DISCUSSION

Collagen has been widely used in medical applications ever since it was first pioneered many years ago by Ehrmann and Gey [Ehrmann and Gey, 1956]. Since those initial studies, collagen has been used to maintain the growth, differentiation and the expression of tissue-specific functions of a wide variety of cells types [Ivarsson *et al.*, 1998; Lynch *et al.*, 1995; Reznikoff *et al.*, 1987]. To overcome the inherent weak mechanical properties, thermal instability and ease of proteolytic breakdown associated with the reprocessing of native collagen; a variety of techniques have been utilised to post-treat and further cross-link the collagen to restore its native characteristics. However, residual chemical agents and impracticality of these techniques at the large-scale have driven the search for alternative and safer techniques.

As such, the focus of this investigation was to exploit a biological catalyst- an enzyme- in order to achieve the desired cross-linking. The transglutaminases (TGs) are a group of enzymes that catalyse a Ca^{2+}-dependent acyl transfer reaction which results in the formation of an ε-(γ-glutamyl)lysine bond between the γ-carboxyl group of a glutamine residue in one polypeptide chain and the ε-amino group of a lysine in a second polypeptide chain [Lorand and Conrad, 1984]. An exception being mTG, a transglutaminase derived from *Sv. mobaraense* and is known to be Ca^{2+}-independent [Ando *et al.*, 1989]. In short, the TGs catalyse the post-translational modifications of proteins and it is this cross-linking reaction that we have exploited throughout this investigation. Moreover, the TGs are able to further catalyse a number of distinct reactions that lead to a post-translational modification of specific glutamine or lysine residues in the target substrate [Folk and Finlayson, 1977] and thus, allow the addition of new properties or molecules to the protein- thereby leading to a possibly enhancement of the substrate function. It has been widely considered that TG-mediated cross-linking could contribute to the design of materials with distinct biological properties by either the direct cross-linking or, by the splicing of TG-related substrate sites onto peptides for subsequent TG-catalysed cross-linking reactions [Chen *et al.*, 2005; Heath *et al.*, 2002; Sato *et al.*, 1996]. As such, bi-functional peptides containing a TG cross-linking site (as well as an RGD cell attachment site) have also been used to alter the biological properties of matrices [Verderio *et al.*, 2001; Schense and Hubbell, 1999]. This strategy not only realises the potential of providing cell attachment sites to a scaffold but also the possibility of employing, via incorporation or by entrapment, available

polypeptide growth and differentiation factors to facilitate matrix resorption, which may yield a constant slow release and prolonged lifetime of the factor, thereby enhancing its biological effectiveness [Aeschlimann and Thomazy, 2000].

As such, we have demonstrated that on treating native collagen type I (and type III) with both tTG and mTG leads to the formation of ε-(γ-glutamyl)lysine cross-links- in contrast to the findings by Jelenska and colleagues [Jelenska *et al.,* 1980]. Accordingly, similar work has also demonstrated that these TGs are capable of cross-linking other collagen fibrils, namely; collagen type V and XI [Kleman *et al.,* 1995], collagen type II [O'Halloran *et al.*, 2006] and some of the non-fibrillar collagens such as type VII [Raghunath *et al.,* 1996] and type X [Lisenmayer *et al.,* 1998]. In addition, other investigatiors have also reported that TG-mediated cross-linking also occurs in the collagens from other species, namely porcine collagen type I [Chen *et al.,* 2005 and 2003], equine network-forming collagen type IV [Walter *et al.,* 2005] and collagen type I derived from shark skin [Nomura *et al.,* 2001].

Although, little is known regarding the exact mechanism of the collagen fibril formation from its monomers, previous work has demonstrated that the kinetics of the *in vitro* collagen fibril assembly consists entirely of two distinct phases: an initiation step that is then followed by a 'growth surge' [Na et al., 1986; Williams *et al.*, 1978; Comper and Veis, 1977; Wood and Keech, 1960] and that the kinetic results of *in vitro* fibril assembly of the TG-treated collagen type I presented here are unanimous with the classical fibril formation process outlined by many previous investigators in the field - albeit at a much quicker rate [Nomura *et al.*, 2001; Na *et al.,* 1986]. It can also be seen that these isopeptide bonds enhance the mechanical/physical and rheological properties of the collagen in such a way that the inherent strength, fibril formation and the gelling characteristics of the substrate improve dramatically. Furthermore, the newly modified matrices are not only capable of absorbing a greater amount of free radicals (that is to say, possessing improved antioxidant ability) compared to native collagen but, additionally, the TG-treated matrices demonstrate a greater resistance to a wide range of proteases. Although it can be seen that native collagen type I naturally contains trace amounts of the ε-(γ-glutamyl)lysine bonds; on treating the collagen with the transglutaminases, a decisive increase in the amount of isopeptide bonds becomes evident and appears to be the sole contributing factor to the demonstrated enhancement to the physical and mechanical behaviour of the

modified collagen. Furthermore, the presence of "background" ε-(γ-glutamyl)lysine bonds in the native collagen implicates that TG may also play a role in normal physiological functions and possibly their associated regulation as documented by several authors [Verderio *et al,* 2004; Griffin *et al.*, 2002; Haroon *et al.*, 1999; Johnson *et al.,* 1999; Upchurch *et al.,* 1991].

Collagen is a very popular biomaterial due to its biocompatibility and has been extensively used as a candidate for artificial skin, tendon, blood vessels, cartilage and bones [Seal *et al.,* 2001]. Yet, although collagen-based materials are still limited in their exploitation due to its inherent weakness- in terms of physical and mechanical strength- these inadequacies have been overcome to a large extent by the introduction of additional cross-linking as described and reviewed earlier. However, it is known that in the majority of cases that involve a modification of the innate structure of native collagen, the resulting product may demonstrate or express retardation in its ability to support cell growth and, indeed, its overall biocompatibility *in vivo* may well be distorted or disrupted [Goo *et al.,* 2003]. Nevertheless, we have now demonstrated that on treating collagen with both tTG and mTG, not only are the physical characteristics improved and enhanced but have also shown that this newly modified substrate, in fact, elicits a greater and more favourable environment for cell maintenance, growth and differentiation. For both HOB and HFDF cells, cultured on the TG-treated collagen matrices, greater and increased spreading and attachment characteristics, proliferation rates, differentiation and migratory behaviour can be noted. Crucially, these parameters occur and are all maintained for the duration of culture without causing or leading to premature cell death (i.e. necrosis or apoptosis) as compared to culture on just native collagen matrices.

As the majority of interactions between cells and the surface of the ECM- or in the case of an applied environment i.e. an implant- occur at the interface, much attention has been focussed on defining and exploiting the characteristics that promote desirable effects [Griffith and Naughton, 2002]. Consequently, with the adhesion of cells to the ECM (or material surface) as the basic initial cellular process that leads to the resultant multitude of cell behaviour, researches have developed novel ways of improving cellular attachment to the biomaterial i.e. by the grafting of synthetic or natural peptides/ligands (that mimic native adhesion molecules) on to the surface of the target substrate. Two of the more commonly used molecules are fibronectin [Mosher *et al.,* 1984] and the RGD adhesion motif

[Pakalns *et al.*, 1999; Grzesiak *et al.,* 1997]. In agreement with the literature, we have confirmed that it is possible to incorporate these ligands to collagen- based on the presence of available substrate residues and, also, report that cellular behaviour and characteristics may be further enhanced due to their incorporation and interaction with the surrounding cells. As such, the only limitation of this technique appears to be as a result of the saturation of residues and/or the possibility of "disruptive" cross-linking; whereby the actual TG-mediated cross-linking may prevent access to other target substrates/residues.

The binding of cells to the ECM are mediated by a group of adhesion molecules known as the integrins and, correspondingly, the α1β1, α2β1, α10β1 and α11β1 integrins have been identified as the dominant collagen receptors [Tuckwell and Humphries, 1993]. An important, yet common, feature for integrin-collagen binding appears to be the reliance on the triple helical structure on binding with the α1β1 and α2β1 integrins- an observation routinely seen in the binding to native collagen rather than to the denatured state e.g. collagen type II [Tuckwell *et al.*, 1994], IV [Vandenberg *et al.,* 1991], V [Ruggiero *et al.,* 1994] and VI [Pfaff *et al.*, 1993]. Gelatin is denatured collagen type I without the 3-D structural ability but still maintains the inherent amino acid sequences of native triple-helical collagen (such as RGD and DGER) to uphold cell growth and survival [Davis *et al.,* 2002]. In this investigation, we confirmed that HOB and HFDF cell-binding to native collagen is mediated by the aforementioned integrins- with their specificity still remaining very dependent on the native collagen structure i.e. binding only occurs in the presence of the triple-helical collagen molecule. However, we interestingly demonstrated that once the native collagen had been treated with either tTG or mTG, cell attachment appeared to occur via a combination of α1β1-, α2β1- and RGD-dependent mediated adhesion- suggesting that TG-modified collagen has been altered in such a manner that it replicates, or is recognised by the cells, as being both native and denatured collagen simultaneously. Confirmation of the structural changes is also apparent as demonstrated by the AFM which shows distinct and unique characteristics of the modified substrates compared to the structure of native collagen. This hypothesis is further reinforced by the cellular response during culture on the quasi-collagen-gelatin matrices.

It is known that the interactions between cells and the ECM underlie a wide range of biological processes and require the successful transduction of cellular signals [Martin *et al.*, 2002; Clark and Brugge, 1995]. As such, the integrin-ligand

interactions dictate and inform the cells as which structural proteins they have encountered before initiating the appropriate gene expression and machinery required to further communicate the downstream processing that relate to the distinct function. Although several complex branches of the multiple signalling pathways that regulate a variety of cellular functions have already been identified; to define the exact flow of a specific signal is a near-impossible task [Assoian, 1997; Ruoshahti and Reed, 1994]. We have demonstrated that cells cultured on the TG-treated collagen matrices express improvements in many of their biological characteristics and that these processes are as a direct consequence of the improved biocompatibility of the modified substrate. As such, and to confirm the abovementioned hypothesis, the distinct MMP expression profiles and precise gene expression of the cells further imply the quasi-nature of the modified collagen. It is increasingly clear that extracellular proteolysis is a tightly regulated process- after all, cells do not release proteases indiscriminately, especially enzymes such as MMP-1, -2, -9 and -13 which possess such a defined substrate specificity- rather, they rely on precise interactions to accurately degrade, cleave or process specific substrates in the pericellular space. However, we report that not only was the collagenase-expression regulated during culture on the matrices but the over-expression of the gelatinases was also apparent.

Furthermore, the gene expression of the cell lines coincided with profiles distinct for cell culture with both collagen and gelatin substrates. Taking into consideration both the physical attributes and the biological features discussed above, it is without a doubt that the modified collagen provides a number of positive features for the cells during their culture and such an influence likely stems from the integrin-mediated recognition of the substrate. Moreover, it is speculated that the observed enhanced physiological processes may be as a result of FAK-mediated cell signalling processes with the relevant and corresponding increase or decrease of FAK-Y397 phosphorylation. Current literature reports and identifies the main collagen receptors and their associated biological processes (see summary in Chapter 5). It can also be seen that such functions are mediated by the same receptors for recognition of collagen. Additionally, the corresponding gene expression profiles are further driven by the same FAK-mediated down stream pathways.

The evidence presented here highlights the fact that various cell-lines prefer TG-treated collagen as opposed to native collagen by the demonstration of a number of enhanced biological processes and cellular functions. It is anticipated and, with a degree of speculation, can be suggested that a possible reason could be that the modified collagen may actually be mimicking an *in vivo* process or, indeed, as described earlier, contributing to the processes that occur during wound healing and tissue remodelling. A more complex process but also involving tissue remodelling is that of bone differentiation. This suggestion is further strengthened by the observed MEF knockout cell-line (which has the tTG gene silenced) that demonstrate enhanced spreading, attachment, proliferation and migration on the TG-modified matrices. Furthermore, TG-cross-linked collagen has been implied in and associated with tumour growth and angiogenesis- two pathological processes that involve cell growth, differentiation and cell survival [Jones *et al.*, 2005].

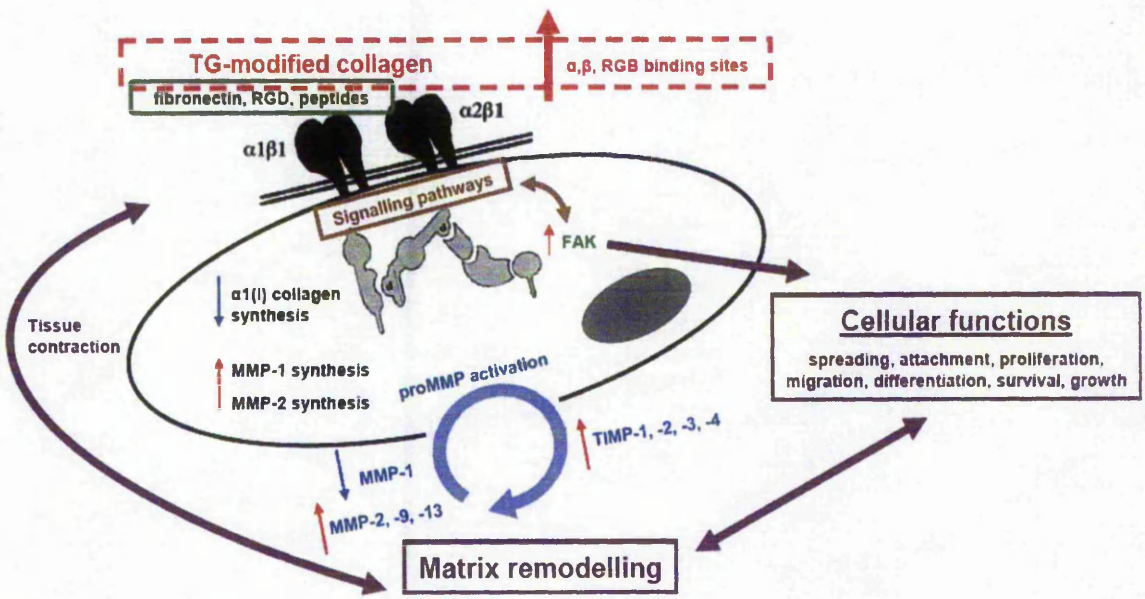


Figure 6.1. Proposed schematic for cellular behaviour on TG-modified collagen matrices.

Hence, the central finding in this thesis is that we have developed a collagen-based biomaterial that possesses all the desired physical properties of an *in vivo* material using a novel and non-toxic technique, and that this substrate expresses enhanced biocompatibility characteristics which can also be further exploited by the addition of other specific adhesion peptides or molecules. Although such a material has not yet entered human clinical trials, the technique has already been

subjected to patent copyright and, currently, our associated collaborators have commenced with several *in vivo* applications. Thus, on summarising, our results suggest that on treating native collagen with the transglutaminases; we not only improve the mechanical properties of the collagen but alter it in such a way that it is perceived differently by the many cell lines during culture. It is thus postulated that this modified collagen presents itself as being native collagen and gelatin simultaneously, yet still maintains the (inherent, if not greater) strength of triple-helical collagen. This behaviour is inferred by the exposure of cryptic binding sites that are characteristic of both native and denatured collagen and, consequently, potentially leads to the required signalling that is mediated by a central protein, FAK, resulting in the downstream processing required for the many observed cellular characteristics such as spreading, proliferation, differentiation and MMP expression and regulation.

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