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TISSUE TRANSGLUTAMINASE & TUMOUR PROGRESSION: CELL DEATH & EXTRACELLULAR MATRIX STABILISATION

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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)

DEDICATION

I would like to dedicate this Thesis to two people:

Firstly, to Stuart Fayle, a good friend who died tragically in a motorbike accident on 14th May 1991, aged 17.

Secondly, to my Nephew, Oliver John Parry, who was born 10 weeks prematurely on 31st March 2002. I wish you all the best in life.

ABSTRACT

The importance of tissue transglutaminase (tTGase, type 2) in tumour progression has been a matter of some debate for a number of years. The role of the enzyme in cell death was investigated following a study carried out some years earlier suggesting that treatment of Met B cells with dexamethasone caused an increase in tTGase within the cells and this was followed by an increase in apoptosis, as measured by the formation of SDS-insoluble bodies. Dexamethasone treatment was shown to induce a powerful, dose-dependent, mRNA synthesis-dependent increase in tTGase activity. The number of SDS-insoluble bodies was found to be comparable with increases in tTGase activity, however, no increase in apoptosis was observed when DNA fragmentation, induction of Caspase-3 and ATP:ADP ratios indicators of classical apoptosis were used. Incubation of cells with the labelled primary amine substrate fluorescein-cadaverine led to its incorporation into cellular proteins following damage resulting from exposure to trypsin during cell passage. The formation of these bodies was shown to be via the activation of tTGase following loss of Ca^{2+} homeostasis rather than induction of classical apoptosis, since Met B cells expressing the bcl-2 cDNA were not protected from SDS-insoluble body formation. It was concluded that a novel type of cell death was taking place which was related to the intracellular activation of tTGase.

Met B cells were further treated with a variety of apoptosis inducing agents both in the presence and absence of dexamethasone. Treatment of the cells with dexamethasone showed no increase in apoptosis, inferring a type of protection.

This new form of cell death was shown to be evident in other cell types (e.g. the P8 rat osteosarcoma), and occurring naturally in organs of normal animals. It was also demonstrated that this type of death could be induced in

tumours, following systemic injections of dexamethasone and intra-tumour injections of ionomycin.

Given the increasing evidence for the involvement of tTGase in cell attachment, migration and extracellular matrix stablisation, work was continued using a partially characterised transplantable rat osteosarcoma (P8). A reduction in tTGase expression in the primary tumour was observed during tumour growth and metastasis. In contrast, secondary tumours showed high levels of expression of tTGase. Immunohistochemical techniques, *in situ* hybridisation and *in situ* activity experiments showed fibrillar patterns of staining of extracellular tTGase, which become reduced as the tumour grows and as tTGase expression drops. Also observed was an increase in the deposition of matrix proteins fibronectin and general collagens, although this may be an accumulation of matrix as the tumour grows, rather than an increase in expression. The limited amount of angiogenesis within the P8 osteosarcoma was also noted.

Cells isolated from the P8 primary tumour were transfected with tTGase cDNA under the control of a constitutive viral promoter. These clones showed increased cell attachment and matrix processing when compared to transfected controls and wild type P8 cells. This is in agreement with current research on tTGase localised at the cell surface. These clones did not form tumours when re-injected back into animals, supporting previous data obtained using other tumour models.

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PUBLICATIONS

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Abstracts

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LIST OF ABBREVIATIONS

ADP	Adenosine-5'-diphosphate
ATP	Adenosine-5'-triphosphate
B4.2	Band 4.2 protein
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
Biotin-X-cadaverine	5(((N-(Biotinoyl)amino)hexanoyl)amino)pentylamine trifluoroacetate salt
bp	Base pairs
Ca^{2+}	Free calcium ion
cDNA	Complementary deoxyribonucleic acid
DAB	3'-5'-Diaminobenzidine
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithoithreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme linked immunosorbant assay
eTGase	Epidermal transglutaminase
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
G418	Geneticin
GDP	Guanosine-5'-diphosphate
GTP	Guanosine-5'-triphosphate
GTPase	Guanosine-5'-triphosphatase
HRP	Horseradish peroxidase
IL	Interleukin

IgG	Immunoglobulin
kDa	Kilodaltons
kTGase	Keratinocyte transglutaminase
LTBP-1	Latent TGF-β_1 binding protein-1
M	Molar
ml	Millilitres
μ	Micro
μl	Microlitres
μM	Micromolar
MOPS	3-(4-morphonyl) 1-propanesulfonic acid
mRNA	Messenger ribonucleic acid
nM	Nanomolar
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
pH	Negative log of hydrogen ion concentration
PMSF	Phenyl methyl sulfonyl fluoride
pTGase	Prostate transglutaminase
SEM	Standard error of the mean
SFM	Serum-free media
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TEMED	N,N,N,N'-tetramethylene diamine
tTGase	Tissue transglutaminase
TGF-β_1	Transforming growth factor β_1
TMB	3,3'-,5,5'-tetramethylbenzidine
TNF	Tumour necrosis factor
Tris	Tris(hydroxymethyl)-aminoethane
Triton X-100	t-Ocylphenoxypolyethoxyethanol

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Chapter 1

INTRODUCTION

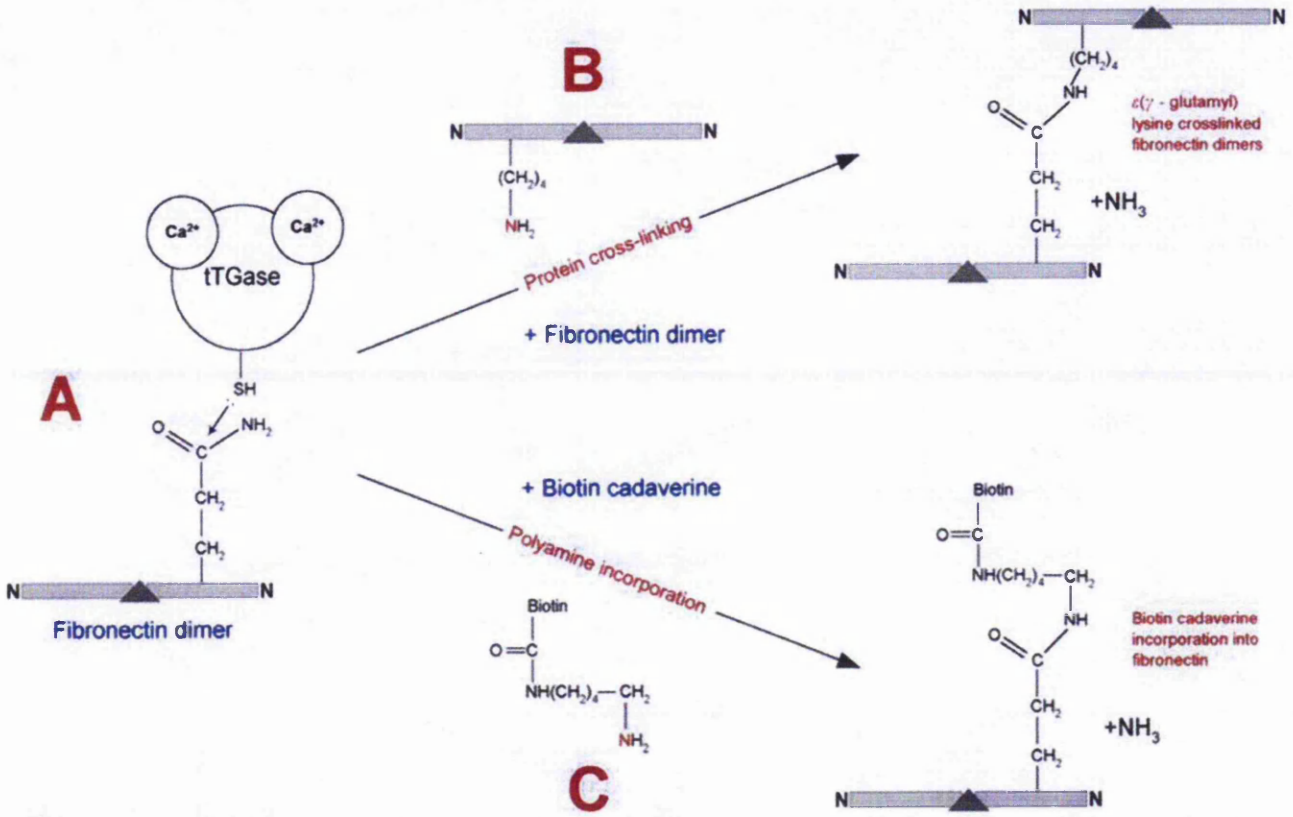
1.1 Transglutaminases

The term transglutaminases was first coined over four decades ago by Clark and co-workers to describe the transamidating activity of an enzyme found in guinea pig liver (Clarke *et al.*, 1957). Since their discovery, many different types of transglutaminases (Enzyme Commission System of Classification 2.3.2.13) have been characterised. All transglutaminases mediate a calcium-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. However, other primary amines can act as substrates in addition to that of peptide-bound lysine e.g. polyamines such as putrescine, spermine and spermidine (Birckbichler *et al.*, 1977, Folk *et al.*, 1980). The types of reaction carried out by transglutaminases are shown in Figure 1.1. The ε-(γ-glutamyl) lysine isopeptide bond formed by transglutaminases cannot be cleaved by any known protease and increase the resistance to mechanical challenge and chemical disruption.

Transglutaminases are widely distributed in nature and are found in a large number of different organisms, implying a functional necessity. Transglutaminase activity has been demonstrated in microorganisms (Kanaji *et al.*, 1993), plants (Serafini_Fracassini *et al.*, 1995), invertebrates (Mehta *et al.*, 1992, Singh and Mehta, 1994) and higher animals including fish (Oppen_Berntsen *et al.*, 1990, Yasueda *et al.*, 1994), amphibians (Zhang and Masui, 1997) and birds (Puszkin and Raghuraman, 1985). In mammals, eight distinct transglutaminase genes for isoenzymes (not including the inactive protein band 4.2 found in erythrocytes) have been isolated from different tissues (see figure 1.2), which are encoded for by different genes, which have been cloned and sequenced (Grundmann *et al.*, 1986, Ikura *et al.*, 1988, Phillips *et al.*, 1990, Gentile *et al.*, 1991, Grant *et al.*, 1994, Aeschlimann *et al.*, 1998, Grenard *et al.*, 2001a).

Figure 1.1: tTGase reaction mechanisms

*Diagram shows protein cross-linking and polyamine incorporation catalysed by tTGase using fibronectin as an example. (A) A peptide bound glutamine residue is subject to nucleophilic attack by the active site Cys277 of Ca2+-bound tTGase,and a ternary complex is formed. The complex may then either react with a peptide bound lysine reside to produce ε(γ-glutamyl) lysine crosslink (B) or react with a primary amine (e.g. biotin cadverine) to produce an ε(γ-glutamyl) polyamide bond (**C**). In both cases, ammonia is released.*


A
B
C
tTGase
Ca2+
Ca2+
SH
NH2
CH2
CH2
N
N
Fibronectin dimer
(CH2)4
NH2
Protein cross-linking
+ Fibronectin dimer
NH
ε(γ - glutamyl)
lysine crosslinked
fibronectin dimers
+NH3
+ Biotin cadaverine
Polyamine incorporation
Biotin
NH(CH2)4—CH2
Biotin cadaverine
incorporation into
fibronectin

Gene name	Gene	Alternate name	Function
F13A1	Factor XIII	Factor XIII, Plasma transglutaminase , fibrin stabilising factor, Laki-Lorand factor, fibrinoligase	Formation & stabilisation of the fibrin clot in the coagulation cascade. ECM stabilisation.
TGM1	TG1	kTGase, transglutaminase type 1, transglutaminase type B keratinocyte-, particulate-, membrane-bound transglutaminase.	Formation of the cornified envelope of terminally differentiating epidermal cells.
TGM2	TG2	tTGase, transglutaminase type 2, $G\alpha_h$, cytosolic-, endothelial-, erythrocyte-, liver-, tissue transglutaminase.	ECM stabilisation, formation of highly crosslinked envelope during cell death, cell signalling.
TGM3	TG3	eTGase, epidermal-, snout-, callus-, hair follicle transglutaminase, transglutaminase type 3.	Formation of the cornified envelope, hair shaft.
TGM4	TG4	pTGase, prostate transglutaminase, transglutaminase type 4, vesiculase, dorsal prostate protein, major androgen-regulated secretory protein.	Formation of the copulatory ring in rodents, semen coagulation.
TGM5	TG5	Transglutaminase X, transglutaminase type 5	Formation of the cornified cell envelope.
TGM6	TG6	Transglutaminase Y, transglutaminase type 6	?
TGM7	TG7	Transglutaminase Z, transglutaminase type 7	?
EPB42	Band 4.2	Erythrocyte protein band 4.2	Structural protein.

Figure 1.2: Table of transglutaminase nomenclature; (Grenard et al., 2001a).

1.1.1 Factor XIII

Factor XIII or plasma transglutaminase is the final zymogen in the blood coagulation cascade (Ichinose and Davie, 1988). The enzyme exists in the plasma in the inactive form as a tetramer of two 83kDa A subunits and two 80kDa B subunits. The A_2-dimeric form of the enzyme is also present in platelets, placenta, monocytes, macrophages and megakaryocytes. Factor XIII is converted to factor XIIIa by thrombin, which cleaves the enzyme to form an A_2 dimer and the 37-amino acid activation peptides (Takagi and Doolittle, 1974), followed by a Ca^{2+}-dependent dissociation of the B subunits (Hornyak and Shafer, 1991). It has been proposed that substrate binding produces a conformational change that activates the enzyme (Yee *et al.*, 1996).

The primary γ-glutamyl donor substrate of factor XIII is fibrin. The enzyme selectively crosslinks the γ-chains of fibrin to form the insoluble fibrin clot. The enzyme also crosslinks the α_2-plasmin inhibitor to the α-chain of fibrin to protect the clot from protease attack (Tamaki and Aoki, 1982). Fibronectin is also a factor XIII substrate (Tamaki and Aoki, 1981), and incorporation of fibronectin into the fibrin clot facilitates cell attachment (Corbett *et al.*, 1997), which is likely to promote wound healing (Grinnell *et al.*, 1980).

Other factor XIII substrates include α_2-macroglobulin (Mortensen *et al.*, 1981), vitronectin (Sane *et al.*, 1988), thrombospondin (Lynch *et al.*, 1987), von Willebrand factor (Hada *et al.*, 1986), factor V (Francis *et al.*, 1986), lipoprotein (a) (Borth *et al.*, 1991), plasminogen activator inhibitor (Jensen *et al.*, 1993) and certain collagens (Mosher, 1984). However, although all of these proteins have been shown to be substrates of factor XIII, the closest relationship appears to be with fibrin (Hornyak and Shafer, 1992). Factor XIIIa binds to the Aα and Bβ chains in the D-domain of fibrinogen in the circulating plasma in a manner separate from its crosslinking activity, which may assist in targeting the enzyme during blood clotting.

1.1.2 Keratinocyte transglutaminase (TGase 1)

Keratinocyte transglutaminase (kTGase) is an intracellular enzyme found in epidermal keratinocytes. The approximately 90kDa enzyme (Phillips *et al.*, 1990) is anchored to the inner face of the plasma membrane through fatty acylation of cysteine residues at the proteins N-terminal region that are linked to palmitic or myristic acid (Chakravarty and Rice, 1989, Rice *et al.*, 1990). The N-terminal region may also be phosphorylated by multiple kinases at specific serine residues (Chakravarty *et al.*, 1990).

kTGase is involved in the squamous differentiation of epithelia, and the enzyme is activated by an influx of Ca^{2+} in the senescent epidermal keratinocytes. The enzyme then crosslinks proteins such as loricrin (Hohl, 1993), keratin (Yaffe *et al.*, 1992), involucrin (Eckert and Green, 1986) and filaggrin (Simon *et al.*, 1996) into a thick protein layer or cornified envelope. Recent work has showed that the kTGase is capable of catalysing the formation of an ester bond between specific glutamyl residues in involucrin and ω-hydroxyceramides, which may anchor the cornified envelope to the lipid envelope on the surface of the keratinocyte (Nemes and Steinert, 1999).

The highly crosslinked cornified envelope is thought to be essential for structural integrity of the upper epidermal layer and therefore important in normal barrier function. Loss of function of the kTGase enzyme through disruptions to the kTGase gene is known to contribute to the icthyosis phenotype (Hennies *et al.*, 1998). The development of knockout mice lacking the kTGase gene reinforced the importance of kTGase, as these mice displayed erythrodermic skin with abnormal keratinisation and died within 4-5 hours after birth (Matsuki *et al.*, 1998). In contrast, inappropriate expression of the enzyme in suprabasal cells of the corneal and conjuctival epithelium has been implicated in the early stages of the pathological keratinisation characteristic of Stevens-Johnson syndrome (Nishida *et al.*, 1999).

1.1.3 Epidermal transglutaminase (TGase 3)

Epidermal transglutaminase (eTGase) is the least understood of the mammalian transglutaminases. It is an intracellular enzyme that requires proteolytic cleavage to be activated. The 77kDa proenzyme is cleaved into the catalytically active 50kDa fragment and a 27kDa carboxyl-terminal peptide (Kim *et al.*, 1990). The protease responsible for the cleavage of eTGase is at present unknown, although there is some evidence to suggest the involvement of the calpain family of Ca^{2+}-activated, neutral cysteine proteases (Kim and Bae, 1998).

Current theories on the role of eTGase are concentrating on an involvement in the formation of the cornified envelope of the epidermis as it is found in terminally differentiated epidermal cells (Martinet *et al.*, 1988, Kim *et al.*, 1990). Other researchers have proposed a role in the formation of the hair shaft (Lee *et al.*, 1993b).

1.1.4 Prostate transglutaminase (TGase 4)

Prostate transglutaminase (pTGase) is an extracellular transglutaminase present in the seminal fluid of rodents. The enzyme is thought to be secreted by the dorsal prostate and coagulative gland and exists as a 150kDa homodimer of a 75kDa protein (Ho *et al.*, 1992). It has been proposed that pTGase crosslinks seminal vesicle proteins (SVP's) such as SVP-1 and SPV-IV to form a copulatory plug (Ho *et al.*, 1992, Seitz *et al.*, 1991).

Recent work has identified a human pTGase. The gene has been mapped to chromosome 3p21.33-p22 (Gentile *et al.*, 1995) and encodes a protein of approximately 77kDa in size (Dubbink *et al.*, 1996). Expression of pTGase in humans is exclusively in the luminal epithelial cells. Concentration and distribution of the enzyme in prostatic fluids and tissues is highly variable between individuals and absent in prostate carcinoma cell lines (Dubbink *et al.*, 1999). Semenogelin I and II, the major seminal vesicle secreted proteins

in human semen have been identified as potential substrates for human pTGase (Peter *et al.*, 1998).

1.1.5 Band 4.2 protein

The cytoskeletal network at the inner face of the erythrocyte plasma membrane contains the inactive transglutaminase, band 4.2 (B4.2) or pallidin. The 77kDa protein shows high homology to other members of the transglutaminase family, but the active site cysteine residue is substituted with alanine (Korsgren *et al.*, 1990). B4.2 is associated with the plasma membrane of erythrocytes by myristylation of a specific N-terminal glycine residue in manner similar to that of kTGase (Risinger *et al.*, 1992), and is believed to interact with anion exchanger band 3, ankyrin and band 4.1 protein (Cohen *et al.*, 1993). B4.2 is also an ATP binding protein in common with tTGase, although the function of this property is uncertain (Azim *et al.*, 1996).

Many non-erythroid cells produce isoforms of B4.2, although the precise function is unclear, although it is thought that the protein may provide essential structural stability. Lack of B4.2 by targeted disruption or mutation is known to cause spherocytosis (Peters *et al.*, 1999, Kanzaki *et al.*, 1997) and hereditary haemolytic anaemia (Hayette *et al.*, 1995).

1.1.6 Transglutaminases X, Y and Z

Recently, three additional members of the transglutaminase family have been discovered and named transglutaminase X (or TGase 5), transglutaminase Y (or TGase 6) and transglutaminase Z (or TGase 7). While the physiological role of these enzymes has still to be determined, their gene localisation, organisation and mRNA expression patterns have already been described (Aeschlimann *et al.*, 1998, Grenard *et al.*, 2001a).

The transglutaminase X gene comprises of ~35kb of genomic DNA, containing 13 exons and 12 introns. This gene also exhibits at least two differently spliced mRNA transcripts in human keratinocytes as detected by

Northern blot analysis (Aeschlimann *et al.*, 1998). Expression studies have shown that full length transglutaminase X is induced during the early stages of keratinocyte differentiation and is differently regulated to that found in other epidermal transglutaminases. It has also been shown that transglutaminase X efficiently crosslinks epidermal substrates such as loricrin and involucrin *in vitro*, suggesting its role in cornified envelope formation *in vivo* (Candi *et al.*, 2001).

Novel transglutaminase Y has been localised on the segment containing the genes encoding tTGase and eTGase on human chromosome 20 (Grenard *et al.*, 2001a).

Another novel transglutaminase, transglutaminase Z, has been discovered in human prostate carcinoma. Both transglutaminase X and transglutaminase Z have had a number of alternative splice variants isolated from this source (Grenard *et al.*, 2001a). A comparative sequence analysis has revealed that structural requirements for transglutaminase activity and calcium binding are conserved and core domain containing catalytic amino acid triad shows a high level of homology with other transglutaminases. In addition, transglutaminase X and transglutaminase Z genes have been localised in tandem with band 4.2 gene on human chromosome 15. Expression of transglutaminase X and transglutaminase Z mRNA has been shown in a wide variety of tissues and expression patterns of both novel transglutaminases overlap with tTGase and to a more limited degree, with eTGase (Grenard *et al.*, 2001a).

1.1.7 Tissue transglutaminase (TGase 2)

tTGase is the most widespread of the mammalian transglutaminases that has been characterised in a number of different cells and tissues (Fesus and Thomazy, 1988, Thomazy and Fesus, 1989), and is the enzyme of study in this thesis.

1.2 Tissue Transglutaminase

Tissue transglutaminase (tTGase, Type 2) is a 77kDa enzyme ubiquitously found in mammalian tissues (Folk and Finlayson, 1977). The enzyme is highly conserved with the complete amino acid sequence for tTGase from guinea pig liver (Ikura *et al.*, 1988), mouse macrophages, human endothelial cells(Gentile *et al.*, 1991) and chicken erythrocytes (Weraarchakul_Boonmark *et al.*, 1992) deduced from the corresponding cDNA sequences, showing approximately 80% homology between them.

1.2.1 Structure of tTGase

Amino acid analysis of guinea pig liver tTGase has revealed that, despite having 17 cysteine residues, the protein contains no disulfide bonds (Ikura *et al.*, 1998). tTGase also does not have any classical hydrophobic leader sequences and although six potential N-linked glycosylation sites have been identified, tTGase is not glycosylated (Ikura *et al.*, 1998). tTGase can undergo posttranslational modification at its terminal regions, where its initiator methionine is removed, followed by N-acetylation of the adjacent alanine residue (Ikura *et al.*, 1998). Two regions rich in glutamine residues around amino acids 450 and 470 have been proposed for the calcium-binding site in order to regulate its activity (Ichinose *et al.*, 1990)

The three-dimensional structure of tTGase has been modulated on the basis of factor XIIIa and red sea bream liver transglutaminase (fish-derived tTGase) and has been predicted to consist of N-terminal β-sandwich domain, a catalytic core and two C-terminal barrels (Noguchi *et al.*, 2001). Studies have also revealed that the N-terminal domain of tTGase is required for protein crosslinking and the core domain is important for the hydrolysis of GTP and ATP (Iismaa *et al.*, 1997). More recently, these authors have localised the GTP-binding domain to a 15-residue segment spanning between amino acids 159-173 in the core domain (Iismaa *et al.*, 2000). Interestingly, the GTP-binding domain of tTGase contains almost all of the conserved tryptophans

of the protein and latest point mutation analysis has revealed that Trp241 was critical for tTGase crosslinking activity (Murthy *et al.*, 2002).

1.2.2 Regulation of tTGase

tTGase is expressed in a wide variety of cells and tissues in a highly regulated manner. Many cells such as endothelial cells, vascular smooth muscle cells, platelets and epithelial cells express the enzyme constitutively and accumulate high levels of active enzyme (Thomazy and Fesus, 1989, Greenberg *et al.*, 1991). In other cells, such as monocytes and tissue macrophages, the basal level of tTGase expression is very low, but is dramatically increased during an inflammatory response (Moore *et al.*, 1984, Murtaugh *et al.*, 1984).

1.2.2.1 Regulation of tTGase gene expression

The tTGase gene promoter is constitutively active in many cell types (Lu *et al.*, 1995). The presence of a consititutively active promoter in a gene subject to several regulatory pathways indicates that important negative or tissue-specific regulatory elements are involved to control the expression of this gene in many cells and tissues (Chen and Mehta, 1999). Figure 1.3 shows a schematic diagram of the structure of the tTGase gene promoter.

One of the methods of regulation of the basal level of the tTGase gene promoter is methylation (Lu and Davies, 1997). These researchers demonstrated a direct correlation between methylation of the CpG-rich region of the human tTGase promoter and decreased transcription of the promoter. Also reported was that *in vivo* demethylation of the tTGase promoter increases tTGase expression.

Figure 1.3: Schematic diagram of tTGase promoter (Aeschlimann and Thomazy, 2000)

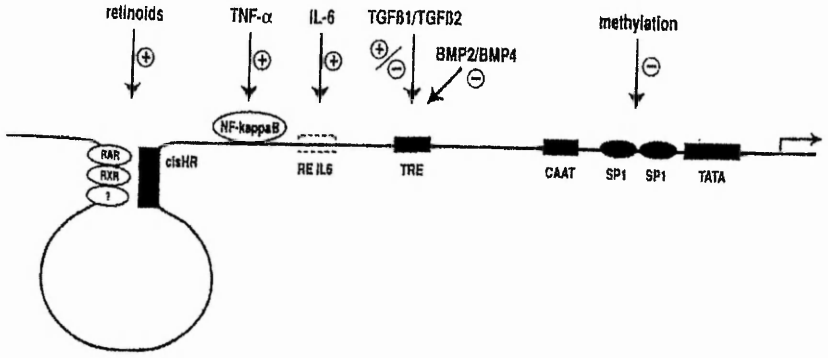


Illustration of the tTGase promoter showing the effects of various factors. The TATA box and upstream SP1 sites provide constitutive expression. Retinoids, TNFa, IL-6 and TGFβ_1 stimulate expression. TGFβ_2, BMP2/BMP4 and methylation inhibit gene expression.

A number of pharmacological agents have been shown to regulate tTGase expression in different cell lines and cell types. These include cyclic AMP (Perry *et al.*, 1995), dimethyl sulfoxide (Hsu and Friedman, 1983), IL-6 (Suto *et al.*, 1993), TNF α (Kuncio *et al.*, 1998), TGF-β1 (George *et al.*, 1990, Ritter and Davies, 1998), sodium butyrate (Byrd and Lichti, 1987, Lee *et al.*, 1987), glucocorticoids (Goldman, 1987) and thrombin (Auld *et al.*, 2001).

However, probably the best characterised pharmacological agent known to effect the tTGase gene promoter is retinoic acid. Retinoic acid has been shown to induce tTGase in several cell lines and types, both *in vitro* and *in vivo*, with increases of mRNA and protein (Davies *et al.*, 1985, Mehta and Lopez_Berestein, 1986, Murtaugh *et al.*, 1986, Chiocca *et al.*, 1988, Verma *et al.*, 1992, Defacque *et al.*, 1995, Zhang *et al.*, 1995). Retinoids exert their biological effects by interacting with two families of intracellular nuclear receptors: the retinoic acid receptors (RAR) and the retinoid X receptors (RXR) (Chambon, 1994). It has been demonstrated that induction of tTGase expression in different cell lines requires ligand-dependent activation of either or both RAR and RXR (Zhang *et al.*, 1995, Joseph *et al.*, 1998). In addition, the retinoid acid induction of tTGase can be altered by factors such as protein kinase activators such as cholera toxins, or reduced by others such as pertussis toxins (Johnson and Davies, 1986, Ishii and Ui, 1994).

1.2.2.2 Regulation of tTGase activity

tTGase is a bifunctional enzyme. The enzyme catalyses Ca^{2+}-dependent transamidating reactions and Ca^{2+}-independent hydrolysis of GTP and ATP (Lee *et al.*, 1989, Lee *et al.*, 1993a).

1.2.2.2.1 The transamidating function of tTGase

The requirement of Ca^{2+} as an activator was recognised as early as 1967 (Folk *et al.*, 1967) and early *in vitro* studies recognised that guanoside nucleotides have a negative effect on the crosslinking action of the enzyme (Achyuthan and Greenberg, 1987).

Inside the cell, tTGase crosslinking activity is tightly regulated by low Ca^{2+} and high GTP concentrations (Smethurst and Griffin, 1996). For tTGase crosslinking to occur, 3-4 Ca^{2+} ions in the absence of GTP, GDP, ATP or ADP (Folk and Finlayson, 1977). The non-competitive binding of these nucleotides promotes a conformational change in the enzyme, inhibiting its activity, although this can be partially reversed by the addition of Ca^{2+} ions (Achyuthan and Greenberg, 1987). The tight regulation of tTGase crosslinking makes it unlikely that the crosslinking activity of the enzyme would ever be active under normal, intracellular physiological conditions (Smethurst and Griffin, 1996).

Other factors have been shown to regulate tTGase activity besides Ca^{2+} and nucleotides. Studies by Lai and co-workers shows sphingosylphosphocholine can serve as a co-factor for tTGase that reduces the Ca^{2+} requirement of the enzyme (Lai *et al.*, 1997). Nitric oxide has also been been shown to modulate tTGase activity by S-nitrosylation of the active site cysteine and the formation of S-nitrosothiol (Melino *et al.*, 1997). More recent studies have indicated that S-nitrosylation and denitrosylation of tTGase is regulated through a unique Ca^{2+}-dependent mechanism where, in the presence of Ca^{2+}, more cysteine residues were nitrosylated, resulting in inhibition of the enzyme. However, this effect could be reversed by the addition of more Ca^{2+} ions, which triggered denitrosylation (Lai *et al.*, 2001).

1.2.2.2.2 The GTPase function of tTGase

As previously stated, in addition to the crosslinking ability of the enzyme, tTGase can also bind and hydrolyse GTP and ATP in a Mg^{2+}-dependent manner (Lee *et al.*, 1989, Lai *et al.*, 1998). Mg^{2+}-GTP and Mg^{2+}-ATP have been shown to be the true substrates for tTGase mediated hydrolysis (Lai *et al.*, 1998). These researchers also showed that Mg^{2+}-ATP induces a conformational change in the enzyme that inhibits GTPase activity, but does not interfere with protein crosslinking activity. In contrast, Mg^{2+}-GTP

binding induces a different conformational change that inhibits protein crosslinking, without affecting the ATPase activity of tTGase. Also demonstrated in this study was that Mg^{2+}-GTP binding protects tTGase from proteolytic digestion by trypsin, whereas Mg^{2+}-ATP has no effect (Lai *et al.*, 1998).

1.2.3 Localisation of tTGase

1.2.3.1 Cytosolic tTGase

tTGase has long been described as a cytosolic enzyme, since this is where the majority of the enzyme is detected. However, as described above, the crosslinking activity of the enzyme would be rendered useless under normal physiological conditions due to the low Ca^{2+} and high GTP and ATP concentrations (Smethurst and Griffin, 1996). The theory that tTGase is a cytoplasmic enzyme is further validated since tTGase does not have a classical hydrophobic leader sequence (Ikura *et al.*, 1988).

However, the presence of the enzyme has been detected in the cell membrane fraction (Griffin *et al.*, 1978, Barnes *et al.*, 1985, Slife *et al.*, 1985, Tyrrell *et al.*, 1986, Juprelle_Soret *et al.*, 1988), nucleus (Singh *et al.*, 1995, Lesort *et al.*, 1998, Peng *et al.*, 1999), and in the extracellular matrix (ECM) (Barsigian *et al.*, 1991, Aeschlimann and Paulsson, 1994, Martinez *et al.*, 1994, Jones *et al.*, 1997, Johnson *et al.*, 1997b, Verderio *et al.*, 1998). Since tTGase has been shown to have the ability to crosslink many cytoskeletal proteins (Cohen *et al.*, 1985, Cohen and Anderson, 1987, Takashi, 1988, Harsfalvi *et al.*, 1991, Huang *et al.*, 1992), it has been suggested that the enzyme may bind to these proteins or be present in this fraction due to autocrosslinking itself to stress fibres (Chowdhury *et al.*, 1997), which may account for the sub-cellular distribution of the enzyme.

1.2.3.2 Membrane-associated tTGase

Sub-cellular fractionation and electron microscopy studies have shown that the majority of tTGase is soluble and recovered in the supernatant fraction of

cell extracts, however, a significant amount of the enzyme is also found associated with the membrane fraction, where it remains, even after extensive washes (Barnes *et al.*, 1985, Slife *et al.*, 1985, Tyrrell *et al.*, 1986, Juprelle_Soret *et al.*, 1988). Transglutaminase extracted from the particulate fraction has been demonstrated to display enzymatic and structural properties closely resembling those of the cytosolic tTGase, indicating that it could be a specific sub-cellular location of the enzyme (Chung *et al.*, 1988). Studies on the particulate fraction of rat liver homogenate have also shown the presence of protein substrates in the plasma membrane, accessible to the membrane associated tTGase and that these substrates form large molecular weight aggregates which are not dissociated by sodium dodecyl sulfate (SDS) and disulfide reducing agents (Slife *et al.*, 1986, Tyrrell *et al.*, 1986, Tyrrell *et al.*, 1988b). Further work has revealed that one of the components of these high molecular weight aggregates is fibronectin (Tyrrell *et al.*, 1988a).

1.2.3.3 Nuclear tTGase

tTGase has also been shown to be present in the nucleus, exhibiting both protein crosslinking and G-protein activity (Singh *et al.*, 1995, Lesort *et al.*, 1998). Sub-cellular fractionation of SH-SY5Y human neuroblastoma cells demonstrated that 7% of the total tTGase was localised in the nucleus. 6% was co-purified with chromatin-associated proteins and the other 1% was found in the nuclear matrix fraction (Lesort *et al.*, 1998). These researchers also showed that tTGase crosslinking activity was very low in the nucleus, but could be activated *in situ* by treatment of the cells with maitotoxin. Analysis of this crosslinking revealed different substrates for tTGase than in the nucleus. Several nuclear substrates have been identified including histones (Ballestar *et al.*, 1996, Shimizu *et al.*, 1997), retinoblastoma protein (Oliverio *et al.*, 1997) and transcription factor SP1 (Han and Park, 2000).

The high molecular weight of tTGase (~80kDa) rules out the possibility of passive diffusion through the nuclear membrane, thus indicating an active

transport mechanism to get the enzyme into the nucleus. Co-localisation of tTGase and importin-α3 has been detected in the cytosol, indicating the possible physiological association of the two proteins *in vivo* (Peng *et al.*, 1999). This would conform with known mechanisms of nuclear transport, whereby importin-α3 remains bound to a transported protein only in the cytosol and rapidly dissociates once the nuclear membrane is crossed (Gorlich *et al.*, 1995, Kohler *et al.*, 1997). The role of tTGase in the nucleus has so far not been addressed.

1.2.3.4 Extracellular tTGase

The extracellular environment provides a much more tTGase-mediated crosslinking friendly surrounding for the main transamidating role of the enzyme to take place. The low nucleotide and high Ca^{2+} concentrations make the extracellular space the most likely sub-cellular location for protein crosslinking to occur. This is reinforced by the discovery that the tTGase has the ability to bind and crosslink several ECM components such as fibronectin (Turner and Lorand, 1989, Barsigian *et al.*, 1991, Martinez *et al.*, 1994), fibrin and fibrinogen (Achyuthan *et al.*, 1988), vitronectin (Sane *et al.*, 1988), osteonectin (Aeschlimann *et al.*, 1995), osteopontin (Kaartinen *et al.*, 1997), laminin-nidogen complex in the basement membrane (Aeschlimann and Paulsson, 1991) and different types of collagen (Juprelle_Soret *et al.*, 1988, Kleman *et al.*, 1995, Esterre *et al.*, 1998).

The involvement of tTGase in ECM stabilisation was first suggested in the formation of the fertilisation envelope in sea urchin eggs (Battaglia and Shapiro, 1988). Since then, tTGase has been implicated to play a role in the stabilisation of many different tissues. Since nidogen is a substrate for tTGase, the basement membrane has been examined and crosslinking found near collagen-rich connective tissue, and tTGase itself found co-localised with nidogen in liver, heart, muscle and kidney tissue (Aeschlimann and Paulsson, 1991). tTGase has also been found to be present in differentiating cartilage.

Here it is thought to crosslink matrix proteins such as osteonectin before mineralization of the tissue (Aeschlimann *et al.*, 1993, Aeschlimann *et al.*, 1995).

More recently, tTGase has been implicated in the storage and regulation of the extracellular pool of latent transforming growth factor β_1 ($TGF\beta_1$) through the incorporation of Latent $TGF\beta_1$ binding protein (LTBP-1) into the ECM (Nunes *et al.*, 1997, Verderio *et al.*, 1999). Since the storage and subsequent release of the ECM-bound Latent $TGF\beta_1$ is recognised as a key mechanism in the remodelling of ECM during wound healing and development (Border and Ruoslahti, 1992, Nakajima *et al.*, 1997), the regulation of the extracellular pool of tTGase may be a key factor in localising and concentrating Latent $TGF\beta_1$ complexes during tissue remodelling (Verderio *et al.*, 1999). Immunohisotchemical data has provided evidence that changes in regulation of tTGase leads to changes in the amounts of extracellular LTBP. These researchers also demonstrated a co-localisation of LTBP-1 with tTGase and fibronectin in the ECM, suggesting that fibronectin may be one of the matrix components that LTBP-1 gets crosslinked to as a result of tTGase activity (Verderio *et al.*, 1999).

1.2.3.4.1 tTGase interaction with fibronectin

Without doubt, the most characterised protein that interacts with tTGase is fibronectin. As well as being a natural substrate for tTGase crosslinking, tTGase has a specific fibronectin binding site distant to the active site of the enzyme (Jeong *et al.*, 1995, Gaudry *et al.*, 1999a). *In vitro*, tTGase binds with high affinity (Kd ~8nM) and 2:1 stoichiometry. This interaction has been shown to be mediated by the 42kDa gelatine-binding fragment of fibronectin that lacks any known integrin binding motifs (Turner and Lorand, 1989, Radek *et al.*, 1993). Rotary shadowing electron microscopy studies of guinea pig liver tTGase/fibronectin complexes showed that the enzyme binds within

5-10nm of the N-terminus of the thin fibronectin strands (LeMosy *et al.*, 1992).

1.3 Proposed roles of tTGase

Given the wide substrate requirement of tTGase and the controversy over its cellular location, the enzyme has been implicated in a number of essential biological processes. The following section will discuss these roles which will be shown to encompass almost all stages of cell growth, development and death. However, many of the methods used to study the enzyme in living cells often produce non-specific or artefactual evidence for the involvement of tTGase, therefore the role of the enzyme remains controversial. Although the existence of tTGase was first reported four and a half decades ago (Clarke *et al.*, 1957), the true biological significance of the enzyme has yet to be discovered.

1.3.1 Receptor-mediated endocytosis

Receptor-mediated endocytosis is the term given to the process by which receptor bound proteins are transported into cells via internalisation of clathrin-coated vesicles. The extracellular Ca^{2+}-dependent clustering of ligand-receptor complexes over clathrin-coated pits was found to be inhibited by primary alkylamines (Maxfield *et al.*, 1979), which suggested the potential involvement of tTGase in the process. The role of tTGase in receptor-mediated endocytosis was first proposed in a study by Daveis *et al.* (Davies *et al.*, 1980), where a diverse range of tTGase inhibitors were found to prevent aggregation of α_2-macroglobulin and its subsequent internalisation by fibroblasts. The inhibitors used include: alkyl amines, glutamyl and lysyl peptides, a peptide antibiotic and a sulphonyl urea. The group retracted some of the claims made in this paper when it came to light that some of the findings may have been due to drug toxicity rather than tTGase inhibition in some of their experiments. It was also discovered that some cells completely devoid of tTGase could completely internalise receptor-bound ligands,

forcing the group to admit that tTGase is probably not essential in this process (Davies and Murtaugh, 1984).

Whilst a role for tTGase in receptor-mediated endocytosis in fibroblasts now seems unlikely, there remains evidence that the enzyme participates in macrophage receptor-mediated phagocytosis. Experiments have been carried out showing activated macrophages exhibit higher tTGase activity than their non-stimulated counterparts. Furthermore, tTGase can make up 1-2% of the total cellular protein of activated macrophages (Leu *et al.*, 1982, Murtaugh *et al.*, 1983). Also, the accumulation of tTGase has been linked to an enhanced capacity for phagocytosis (Schroff *et al.*, 1981) and tTGase inhibitors such as cystamine, methylamine and dansylcadaverine have been shown to block Fc-mediated endocytosis (Fesus *et al.*, 1981, Leu *et al.*, 1982). The suggested mode of action is that tTGase processes the Fc receptors into a high affinity state promoting phagocytosis (Davies and Murtaugh, 1984), although the exact mechanism by which this could occur remains unresolved.

1.3.2 tTGase-mediated insulin secretion

Early studies by Bungay *et al.* (Bungay *et al.*, 1984) showed that treatment of rat islets with competitive primary amine substrates of tTGase leads to an inhibition of glucose-stimulated insulin release. The authors suggested that primary amines might inhibit insulin release by incorporation of these amines into crosslinking sites by islet tTGase. Further investigation of the endogenous substrate proteins of islet tTGase revealed highly crosslinked transglutaminase-mediated polymeric aggregates in the 71,000 x g sedimented material of homogenates. This suggested to them the involvement of islet tTGase in the glucose-stimulated release of insulin from prancreatic islets (Bungay *et al.*, 1986), proposing that tTGase participates in controlling the access of secretory granules to the exocytotic sites of pancreatic B cells (Sener *et al.*, 1984).

Latest studies have demonstrated glucose intolerance with hyperglycemia due to reduced insulin secretion in tTGase -/- transgenic knockout mice (Melino *et al.*, 2001). These authors also reported tTGase knockout mice had a tendency to develop hypoglycaemia after administration of exogenous insulin.

1.3.3 Regulation of cell growth

Studies using human fibroblasts have demonstrated that cell growth is initiated in the presence of tTGase inhibitors and arrested in the presence of compounds known to induce tTGase (Birckbichler *et al.*, 1981). They proposed that crosslinking is minimised in proliferating cells and increased tTGase activity serves to stabilise cells and arrest growth. This theory was used to explain previous findings that simian virus transformed WI-38 human lung fibroblasts exhibited a 98% reduction in tTGase activity and content which was accompanied by a dramatic increase in their proliferative capacity (Birckbichler and Patterson, 1978). However, more recently, transfection studies have shown cell lines that overexpress tTGase exhibit similar rates of growth to their parent line and sham-transfected counterparts (Gentile *et al.*, 1992, Johnson *et al.*, 1994, Verderio *et al.*, 1998).

Other evidence suggests that tTGase may indirectly influence cell growth through its affect on growth factors. tTGase is known to be a component in the cell-surface, plasma-mediated activation of TGFβ (Kojima *et al.*, 1993), a potent growth inhibitor. Dimerization of the growth factor midkine by tTGase has been shown to increase its potency (Kojima *et al.*, 1997). It has also been suggested that tTGase may inhibit the growth signal of epidermal growth factor (EGF) in hepatocytes through the down-regulation of its high affinity receptor (Katoh *et al.*, 1996).

1.3.4 tTGase as a GTP and ATP-binding protein

tTGase has been shown to be able to bind and hydrolyse GTP and ATP (Lee *et al.*, 1989, Iismaa *et al.*, 1997). The discovery that tTGase is a fully functional GTPase and ATPase considerably complicates the search for a true biological

role. Given the stringent controls on the protein crosslinking function of the enzyme and the unlikelihood of crosslinking occurring normally in the cell, it is possible that the enzymes primary role may be as a cell signalling protein.

GTP binding protein G_h was co-purified from ternary complexes containing the agonist bound α_1-adrenergic receptor (Im *et al.*, 1997). G_h was found to be a heterodimer made up of a 74kDa, GTP-binding α-subunit ($G\alpha_h$) and a 50kDa β-subunit ($G\beta_h$) that modulates nucleotide binding and hydrolysis by $G\alpha_h$ (Im *et al.*, 1997, Baek *et al.*, 1996). Microsequence analysis of $G\alpha_h$ peptide fragments revealed it to be tTGase. Transfection studies with tTGase and α_{1B}-adrenergic receptor cDNA indicated that tTGase was the G-protein involved in α_1-adrenergic stimulation of phospholipase C(Nakaoka *et al.*, 1994). Further research into this signalling pathway showed that $G\beta_h$ co-immunoprecipitates with GDP-bound tTGase, but becomes dissociated from GTP-bound tTGase. $G\beta_h$ was found to down-regulate $G\alpha_h$ function by inhibiting GTP binding and thus GTPase activity in a concentration dependent manner. Also, $G\beta_h$ accelerated the release of GTP from $G\alpha_h$ and altered its affinity from GTP to GDP. Studies using the ternary complex containing α_1-adrenergic receptor, agonist and G_h indicated that the receptor enhances the affinity of tTGase for GTP (Baek *et al.*, 1996). The effector in this pathway may be phospholipase C-δ1 (PLC-δ1). An 8 amino acid region in tTGase (Leu^{665}-Leu^{672}) was found to interact with PLC-δ1. Antibodies to tTGase were also found to co-precipitate with PLC-δ1 upon G_h activation (Feng *et al.*, 1996).

Although most of the work carried out on G_h has focussed on its association with the α_1-adrenergic receptor, some groups have found that G_h may also interact with the oxytocin receptor in human myometrium (Baek *et al.*, 1996) and certain thromboxane receptors (Vezza *et al.*, 1999). Another group of researchers looking for nuclear GTP-binding proteins discovered an 80kDa protein responsible for the majority of GTP-binding activity in rabbit liver

nuclear extracts (Singh *et al.*, 1995). Microsequence analysis showed this to be tTGase too. Co-purified with tTGase was a 36kDa GTP-binding protein proposed to be an N-terminal proteolytic fragment of tTGase. This peptide exhibited a significantly higher binding affinity for GTP than the intact tTGase. Due to the difficulties associated with extracting the peptide lead the group to speculate that the protein was a component of the nuclear pore/lamina. This hypothesis was supported by a similar distribution of tTGase with nuclear pore specific protein (p62) by immunofluorescence. This finding was reinforced by immunoprecipitation experiments in which an anti-p62 antibody co-precipitated with tTGase from rabbit liver nuclear preparations (Singh *et al.*, 1995). Continuing this work, the group went on to claim that retinoic acid stimulated GTP incorporation into tTGase in HeLa cells. Untreated cells were reported to form a high molecular weight form of the enzyme, deficient in both protein crosslinking and GTP-binding abilities. Retinoic acid treatment was shown to promote association with the plasma membrane, which could be correlated to stimulation of membrane-associated PLC activity (Singh and Cerione, 1996), giving further evidence of tTGase involvement in the PLC signalling pathway.

1.3.5 tTGase and the cytoskeleton

Three main types of fibres maintain the internal architecture of the cell: microfilaments, microtubules and intermediate filaments. Many cytoskeletal proteins have been shown to be tTGase substrates *in vitro*, including actin and myosin (Eligula *et al.*, 1998, Kang *et al.*, 1995), tubulin (Cohen and Anderson, 1987), α-actinin (Puszkin and Raghuraman, 1985) and spectrin (Harsfalvi *et al.*, 1991), although it is uncertain whether crosslinking occurs *in vivo* under normal physiological conditions, this may be important in cell death, which is discussed later in this chapter. However, evidence of tTGase crosslinking of cytoskeletal elements such as band 3, band 4.1, ankyrin and spectrin has been detected in ageing erythrocytes (Lorand and Conrad, 1984).

Some studies have suggested specific associations with cytoskeletal components. In dermal fibroblasts, tTGase was found to co-localise with vimentin intermediate filaments (Trejo_Skalli *et al.*, 1995), which was maintained when cells were treated with colchicine, which causes microtubule disassembly and the reorganisation of intermediate filaments into perinuclear aggregates. Furthermore, microinjection of cells with an anti-tTGase antibody was found to cause a collapse of the vimentin intermediate filament network in live fibroblasts.

Other studies have suggested tTGase associates with myosin-containing cytoskeletal structures. One such group reported that tTGase may be a component of myofibril assembly (Kang *et al.*, 1995), showing tTGase co-localising with the cross-striated sarcomeric A band of myosin thick filaments during the maturation of chick embryonic myoblasts and suggested that myosin crosslinking was an essential part of myofibrillogenesis. Another study reported co-localisation of tTGase with stress fibre-associated myosin in human vascular smooth muscle cells and human umbilical vein endothelial cells (Chowdhury *et al.*, 1997). Here, tTGase was found to co-immunoprecipitate with myosin in high molecular weight complexes, leading the authors to believe that the enzyme autocrosslinks itself to stress fibre-associated myosin.

1.3.6 tTGase in cell attachment and adhesion

As detailed earlier in this chapter, despite the lack of a classical leader sequence, the presence of tTGase on the cell surface and in the ECM is well documented (Barsigian *et al.*, 1991, Martinez *et al.*, 1994, Aeschlimann and Paulsson, 1994, Aeschlimann *et al.*, 1995, Jones *et al.*, 1997, Verderio *et al.*, 1998, Verderio *et al.*, 1999, Akimov *et al.*, 2000). Increasing evidence suggests that tTGase has an important role in cell attachment and adhesion. Early studies by Gentile *et al.* (Gentile *et al.*, 1992) showed fibroblasts overexpressing tTGase demonstrated an increased cell spreading and reduced

susceptibility to detachment by trypsin. Reductions in expression of tTGase or inactivation of the cell surface enzyme by targeted antibody have shown decreased cell adhesion and spreading in different cell lines (Jones *et al.*, 1997, Verderio *et al.*, 1998).

The interaction of cells with the ECM is mediated through the action of cell surface receptors such as integrins. Since integrins are not always reactive with ligand molecules, various modulators have been suggested (Isobe *et al.*, 1999). These authors demonstrated *in vitro* cell adhesion is mediated by tTGase by its association with the $\alpha_4\beta_1$-integrin rather than by virtue of its crosslinking ability. Other immunohistochemistry and electron microscopy studies have demonstrated tTGase concentrated to cell adhesion points rich in the β_1-integrin in cells undergoing attachment and spreading (Gaudry *et al.*, 1999b). tTGase has been shown to react with β_3-integrin, forming complexes inside the cell during their biosynthesis, suggesting that integrins could be involved in the transport of tTGase across the plasma membrane and to the cell surface or ECM (Akimov *et al.*, 2000). These authors and others showed overexpression of tTGase leads to an increased amount of cell surface and ECM tTGase. This in turn enhances cell adhesion and spreading on a fibronectin fragment lacking the integrin binding sequence (Verderio *et al.*, 1998), suggesting a separate cell adhesion pathway independent of classical integrin binding, but using tTGase as an integrin-binding co-receptor for fibronectin (Akimov *et al.*, 2000).

1.3.7 tTGase and tissue repair

It has long been suspected that tTGase has a role in the wound healing process. Early studies using rat skin reported an increase in tTGase activity and antigen levels around biopsy punch wounds (Bowness *et al.*, 1987, Bowness *et al.*, 1988). Increased activity has also been demonstrated in paraquat damaged rat lungs (Griffin *et al.*, 1979). It is thought a key tTGase substrate in the repair process may be the aminopropeptide of collagen type

III (Bowness, 1987), which is synthesised during wound healing (Kurkinen *et al.*, 1980).

More recently, tTGase has been implicated in the crosslinking of the papillary dermis and the dermo-epidermal junction during the healing of autografts of burn victims (Raghunath *et al.*, 1996). Monodansylcadaverine was incorporated into the epidermis, dermal connective tissue, along the basement membrane and anchoring fibrils and transamidation was completed within 4-5 months after grafting. This study identified collagen VII as a key substrate and the authors speculated that the topical application of purified tTGase to wounded tissue may accelerate the healing process. The use of tTGase in experimental repair of rat optic nerves has been reported (Eitan *et al.*, 1994) and it is hoped the enzyme will be of value in treating human injuries. Indeed, it is expected a commercially available tTGase-based biological glue will soon be on the market for cartilage-cartilage interfaces. A recent study reported the adhesive strength was superior to that of the more traditionally used fibrin sealant (Jurgensen *et al.*, 1997).

1.4 The role of tTGase in cell death

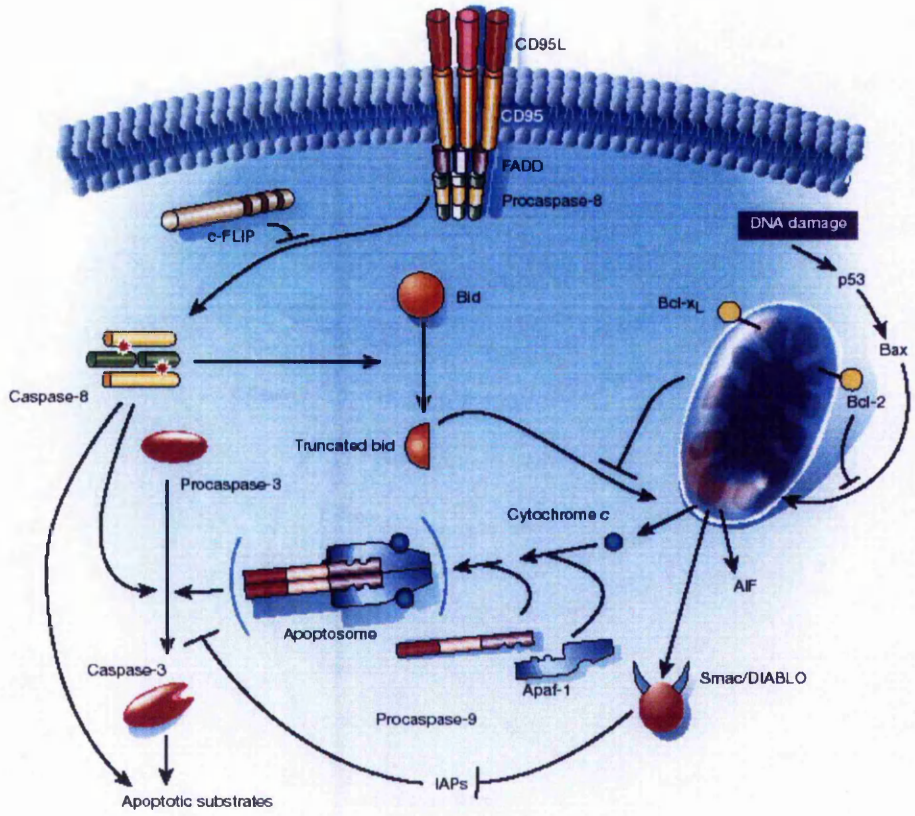
1.4.1 Cell death mechanisms

Tissue homeostasis is balanced by controlling cell division and cell death, as well as by repair mechanisms. Cell death is as important for tissue turnover as the development of new cells. Cell death can occur in two distinct and different pathways: apoptosis (or programmed cell death) and necrosis (which occurs after acute physical or chemical damage).

Necrosis can be observed in tissue areas following acute physical or chemical insult leading to disruption of the cell membrane resulting in complete loss of membrane integrity, cell swelling and leakage of intracellular components into the extracellular environment. Necrosis induces an immune response in the tissue, giving rise to inflammation.

During apoptosis or programmed cell death, cells die in response to a specific physiological signal. Apoptosis was first described by Kerr and co-workers (Kerr *et al.*, 1972) and is known to play a very important role in a number of biological processes including embryogenesis, tissue remodelling (Guenette *et al.*, 1994) and tumour regression (Wyllie *et al.*, 1980). Under physiological conditions, apoptosis requires energy and de novo synthesis of various genes (Arends and Wyllie, 1991, Fesus *et al.*, 1991). Apoptosis can be indueced in several ways and take different forms. The main two apoptotic pathways are caspase-dependent or –independent. These pathways tend to depend on the stimulus for the cell death (Figure 1.4). The death-receptor pathway is triggered by members of the death receptor superfamily, such as CD95 and Tumour Necrosis Factor receptor I. Binding of CD95 ligand to CD95 induces receptor clustering and the formation of a death-inducing signalling complex (DISC). This complex recruits, via the adapter molecule Fas-associated death domain protein (FADD), multiple procaspase-8 molecules, resulting in caspase-8 activation through induced proximity (Hengartner, 2000). Caspase-8 activation can be blocked by recruitment of the degenerate caspase homologue c-FLIP (Kataoka *et al.*, 2000). The mitochondrial pathway is the other apoptotic pathway, used extensively in response to extracellular cues and internal insults such as DNA damage (Rich *et al.*, 2000). These diverse stimuli converge on to mitochondria, often through the activation of a pro-apoptotic member of the

Figure 1.4: Apoptotic pathways



Schematic diagram of caspase – dependent and – independent apoptosis (Hengartner, 2000).

Bcl-2 family. Unlike Bcl-2, which seems to spend most, if not all of its life attached to the intracellular membranes, many group II and group III members, including Bax, Bad, Bim and Bid, can shuttle between the cytosol and organelles (Gross *et al.*, 1999, Li *et al.*, 1998, Wolter *et al.*, 1997, Puthalakath *et al.*, 1999). The cytosolic forms represent pools of inactive, but battle-ready proteins. Pro-apoptotic signals redirect these proteins to the mitochondria, where the fight for the cells survival will take place. Activation of pro-apoptotic members can occur through proteolysis, dephosphorylation and possibly several other mechanisms (Adams and Cory, 1998, Antonsson and Martinou, 2000). If the apoptotic signal is strong enough, the pathway is propagated by the release of cytochrome *c*, which associates with Apaf-1 and then procaspase-9 and calpains to form the apoptosome. Heat sock proteins have also been shown to play a significant role in this process (Jaattela, 1999, Xanthoudakis and Nicholson, 2000). The two pathways converge with the activation of caspase-3, although caspase-independent apoptosis has been observed, thought to be mainly due to the activation of the calpain family of proteases (Earnshaw *et al.*, 1999, Wang and Lenardo, 2000). Caspase-3 activation and activity is antagonised by the IAP proteins, which themselves are antagonised by the Smac/DIABLO protein released from the mitochondrial compartment. Caspase / calpain activation then results in the degradation of the cell by the cleavage of several cellular substrates, such as actin (causing the loss of cell structure) and DNAse I (which is activated to facilitate the cleavage of DNA and production of the fragments giving rise to the DNA ladder) (Hengartner, 2000). Membrane-blebbing occurs towards the end of the apoptotic pathway, with the formation of "apoptotic bodies". These apoptotic bodies are phagocytosed by neighbouring cells or macrophages, preventing the release of toxic cell contents and potentially very damaging genetical material from the damaged cells (Bellamy *et al.*, 1995).

tTGase has been hypothesised to play a role in both types of death, but its roles are unclear. The following section gives an overview of proposed roles for tTGase in cell death programs.

1.4.1.1 tTGase and necrosis

(Lorand *et al.*, 1975) were the first to describe a role of tTGase in cell ageing / death in the red blood cell. Gilad and co-workers followed up this research and documented the increase in endogenous and exogenous tTGase during necrotic processes in injured rat sympathetic nerve ganglia (Gilad *et al.*, 1985). They noticed that following axotomy, tTGase activity rapidly and transiently increased in the ganglion and that the elevation was dependent on changes in calcium levels.

Liver cirrhosis is a severe disease associated with heavy drinking causing inflammation, liver cell necrosis and the appearance of Mallory bodies in hepatocytes. Mallory bodies are filamentous cytoplasmic inclusions which contain insoluble high molecular weight proteins. It has been suggested that tTGase crosslinking of proteins plays a major role in the formation of Mallory bodies in liver and hepatoma cells (Zatloukal *et al.*, 1992). Similar results were obtained from studies on liver and hepatoma cells where elevated levels of crosslinked protein polymers were detected in coagulative necrosis, suggesting tTGase crosslinking of cytokeratin polypeptides in liver and hepatoma cells is closely associated with the process of cell degeneration and death (Fukuda *et al.*, 1991).

1.4.1.2 tTGase and apoptosis

The involvement of tTGase in apoptosis was first suggested by Fesus *et al.* (Fesus *et al.*, 1987). Since then, a number of studies have suggested a role for tTGase in the apoptotic pathway. These roles have been dependent on the formation of highly crosslinked detergent-insoluble bodies to being a key factor in the onset of the apoptotic pathway (Fesus and Thomazy, 1988, Fesus *et al.*, 1989, Fesus *et al.*, 1991, Knight *et al.*, 1991, Jiang and Kochhar,

1992, Piacentini *et al.*, 1992, Piacentini and Melino, 1994, Nagy *et al.*, 1996, Nemes *et al.*, 1996, Melino *et al.*, 1994).

The tTGase gene promoter has been shown to be activated by several agents known to cause apoptosis in certain cell types, such as: retinoic acid (Davies *et al.*, 1985), IL-6(Suto *et al.*, 1993), and TGFβ (Jetten *et al.*, 1986, Melino and Piacentini, 1998).

Apoptosis can be morphologically characterised by the fragmentation of the cell into smaller bodies that are phagocytosed and digested by neighbouring cells. Given this fact, it seems curious that tTGase is activated, as the ε-(γ-glutamyl) lysine isopeptide bonds formed by the enzyme are highly resistant to degradation and the presence of large protein aggregates in the apoptotic bodies would considerably hinder its disposal. Similarly, the cell sized, tTGase crosslinked "apoptotic bodies" identified by Fesus *et al.* (Fesus *et al.*, 1989) seem to be inconsistent with the unobtrusive nature of this form of cell death.

However, despite these inconsistencies, the involvement of tTGase in the apoptotic pathway remains a very popular area of research. This is largely due to the fact that tTGase levels are elevated during apoptosis (Fesus *et al.*, 1987) and the enzyme has so many intracellular substrates, such as actin (Nemes *et al.*, 1997), retinoblastoma protein (Oliverio *et al.*, 1997), myosin (Kang *et al.*, 1995), and β-tubulin (Piredda *et al.*, 1999). Under normal conditions, the enzyme would not be active, but in the high Ca^{2+} concentrations found at the later stages of apoptosis, the enzyme would be crosslinking the majority of proteins in the cytoplasm.

1.5 Disease states associated with tTGase

Given the wide variety of roles attributed to tTGase, it is not surprising that the enzyme is associated with a number of disease states. The following

section will discuss some of these conditions which range from fibrosis to the development of the malignant phenotype.

1.5.1 tTGase and fibrosis

tTGase was first implicated in the progression of fibrosis over two decades ago by Griffin *et al.* (Griffin *et al.*, 1979), who used a paraquat-induced pulmonary fibrosis model in rats. The enzyme is also been implicated in the development of liver cirrhosis (Grenard *et al.*, 2001b), as high amounts of ε-(γ-glutamyl) lysine isopeptide bonds have been detected in the highly insoluble matrix found in Mallory bodies (Zatloukal *et al.*, 1992). Recent evidence has also suggested that tTGase may play a role in the development of kidney fibrosis. Johnson *et al.* (Johnson *et al.*, 1997b, Johnson *et al.*, 1999) demonstrated that increasing tTGase activity and crosslink formation mirrored the progression of renal scarring in the subtotal nephrectomy model of kidney fibrosis in rats. Given the number of substrates available and the conditions to facilitate protein crosslinking, it is not surprising that accidental tTGase release from cells would cause undesirable protein crosslinking leading to fibrosis of the tissue.

1.5.2 tTGase and neurodegenerative disease

tTGase has been proposed in playing a number of roles in the development of neuronal disorders. So far, eight neurodegenerative diseases have been identified that are caused by expansions in $(CAG)_n$ repeats is various genes. This in turn produces proteins with abnormal polyglutamine (Q_n) sequences (Cooper *et al.*, 1999). Peptides containing Q_n domains are very good substrates for tTGase (Kahlem *et al.*, 1996). The Q_n domains can be crosslinked to themselves or to other polyamines, which provide excellent substrates for attachment to another Q_n domain by tTGase (Gentile *et al.*, 1998). The presence and accumulation of polyglutamine-containing protein aggregates within the cytosol and nuclei of affected neurons may lead to neuronal cell death and progressive neurodegeneration (Karpuj *et al.*, 1999).

tTGase has been found in elevated levels in cells from Huntingdon's disease (HD) brains and has been suggested to play a role in the pathology of the disease (Lesort *et al.*, 1999). The formation of nonamyloidogenic nuclear inclusions found in the HD brain are thought to be due to tTGase-mediated protein crosslinking (Karpuj *et al.*, 1999). These authors went on to describe the administration of tTGase competitive inhibitor, cystamine, as potential therapies for HD and other Q_n diseases and showed transgenic mice with HD-like disease showing extended survival rates and reduced symptoms of the disease such as abnormal movements, tremors and weight-loss (Karpuj *et al.*, 2002).

It has been demonstrated that tTGase is increased in Alzheimer's disease and it has been suggested that the enzyme could be partly responsible for the formation of neurofibrillary tangles (Johnson *et al.*, 1997a, Zhang *et al.*, 1997, Zhang *et al.*, 1998). More recently, tTGase has been shown to localise with the tau protein in the neurofibrillary tangles. Since tau is an *in vitro* tTGase substrate, it has been hypothesised that tTGase crosslinking/polyamination of the tau protein alters its metabolism, leading to the pathogenesis of Alzheimer's disease (Tucholski *et al.*, 1999).

Recent reports have also shown that like many genes, particularly those highly expressed in the CNS, tTGase gene can undergo alternative splicing. This can lead to the appearance of a short-form of tTGase lacking the GTP-binding site (Citron *et al.*, 2001). Elevated expression and alternative splicing resulting in this short-form tTGase, not regulated by GTP and therefore more susceptible to fluxes in intracellular Ca^{2+} concentration, have been associated with augmented neuronal loss in affected regions of the demented brain (Citron *et al.*, 2001, Citron *et al.*, 2002). This may represent a further mechanism whereby tTGase contributes to the pathologies of various neurodegenerative diseases.

1.5.3 tTGase and coeliac disease

Over the past five years, tTGase has been recognised as a key autoantigen of the gluten-derived immune response in coeliac disease patients (Dieterich *et al.*, 1997, Molberg *et al.*, 1998, van_de_Wal *et al.*, 1998, Arentz_Hansen *et al.*, 2000, Sollid, 2000). A typical feature of coeliac disease is the appearance of disease-specific IgA autoantibodies in the bloodstream, some of which are targeted against tTGase (Dieterich *et al.*, 1997, Sulkanen *et al.*, 1998). Since the correlation of tTGase autoantibodies and the onset of disease were shown, work has focussed on how tTGase is involved in the disease and improvements on the diagnosis of disease progression (Bazzigaluppi *et al.*, 1999).

Coeliac disease is caused by an uncontrolled T-cell response to gluten peptides in the gut. Accumulating evidence shows may tTGase play a role in the formation of gluten T cell epitopes in the coeliac lesion. Since gliadin is a preferred substrate of tTGase, it may give rise to novel antigenic epitopes (Dieterich *et al.*, 1997). It has also been suggested that deamidation of certain glutamine residues in gliadin by tTGase may activate T cells and contribute to the development of gluten intolerance (Molberg *et al.*, 1998).

To date, 5 gluten peptides have been identified that stimulate T cell clones. The deamidation of these gluten peptides by tTGase is either required, or enhances, T cell recognition of four of these peptides (Molberg *et al.*, 1998, Sjostrom *et al.*, 1998, van_de_Wal *et al.*, 1998, Quarsten *et al.*, 1999, Anderson *et al.*, 2000, Arentz_Hansen *et al.*, 2000). The deamidation of glutamine residues is negligible at physiological pH, but occurs more effectively with increased protonation of amines in an acidic environment (Mycek and Waelsch, 1960). The deamidation of gluten can take place in the stomach, or alternatively can be catalysed by tTGase. The addition of tTGase inhibitor cystamine during the gliadin challenge, often resulted in T cell lines with abolished or reduced responses to deamidated gliadin (Molberg *et al.*, 2001).

1.6 Cancer

Cancer is the second largest cause of death in the Western civilisation. In Britain, one in three people will have cancer diagnosed at some time during their life and one in four will eventually die from the disease (Office for National Statistics, England and Wales, 1997).

Cancer is a multi-genetic disorder which arises in a tissue when the regulatory mechanisms that control cellular proliferation and/or cell death (homeostasis) go awry and uncontrolled growth begins, resulting in the development of a primary tumour. Cells from this primary tumour often detach themselves into general circulation around the body to establish secondary tumours or metastases. These tumours eventually result in a loss of tissue stability, reduced tissue and organ function and often death (De_Flora *et al.*, 2001).

1.6.1 Tumour growth and metastasis

Primary tumours arise due to a series of mutations in genes critical for the regulation of growth and death of a cell. Once these gene mutations result in ineffective protein products, cells can either start to divide rapidly, or fail to die at the appropriate time. In both cases, a mass of tumour cells is the result.

In order for a solid tumour to continue to grow beyond a diameter of approximately 200μm, it usually requires an integrated blood supply in order to supply the interior of the tumour with nutrients and oxygen and carry away metabolic waste products (Dvorak, 1986). To facilitate angiogenesis (the formation of new blood vessels), the tumour releases chemoattractants (such as angiotropin, angiogenin, basic fibroblast growth factor, VEGF and TNFα) that stimulate the migration of the capillary endothelium from the surrounding normal tissue into the tumour (Furcht, 1986). Angiogenesis also provides a route for detached tumour cells to enter the circulatory system.

The next stage in tumour escape is the detachment of tumour cells from the primary tumour, followed by their invasion into the vascular system. Studies

have shown that tumour cells produce proteolytic enzymes (matrix metalloproteases) in order to destabilise the ECM and enter the blood stream (Liotta and Kohn, 1990). However, the organs to which tumours metastasise is not a random process as certain primary tumours show a bias to form secondary tumours in specific areas (Hager *et al.*, 1997).

Once the tumour cells have reached their destination, colonisation of the tissue must occur in order for a new tumour to grow. This involves leaving the blood stream and the laying down of new ECM on which to grow (Hager *et al.*, 1997).

1.6.2 The role of tTGase in tumour growth and development

Given the roles of tTGase in cell death, growth/proliferation and in the ECM, it is not surprising that the importance of the enzyme in tumour growth has been the subject of much research for many years.

Early work by Yancey and Laki (1972) implicated TGases in tumour growth. They proposed that solid tumours required a covalently stabilised fibrin network for proliferative growth. Later, several studies demonstrated that tTGase activity is reduced in neoplastic cells compared to their normal counterparts. (Birckbichler and Patterson, 1978) showed that chemically- and virally-transformed cells contained less tTGase than their normal counterparts. Their follow-up study reported that levels of isopeptide cross-links were dramatically reduced in malignant cells, and that normal cells contained a greater amount of plasma membrane-associated tTGase than transformed cells (Birckbichler and Patterson, 1980). This is in direct contradiction of their previous finding that the majority tTGase activity in normal cells was associated with the soluble cytosolic fraction (Birckbichler *et al.*, 1976). In this *in vivo* study using normal and malignant liver tissue, they showed that the distribution of tTGase in neoplastic tissue changes to become predominantly associated with the particulate fraction. A similar finding was reported by Barnes *et al.* (1985). This group examined tTGase

activity in normal tissue and in a chemically induced osteosarcomas. Enzyme activity in all tested transplantable rat sarcomas was greatly reduced when compared to the corresponding normal tissue, and a further reduction was noted in sarcomas P7 and P8 following detection of metastases. Reduction of tTGase activity was accompanied by a redistribution of the enzyme to the particulate fraction, which was confirmed in a follow-up study (Hand *et al.*, 1988). The involvement of tTGase in metastasis was further investigated using the P8 sarcoma, and at approximately 30 days after implantation the decrease in protein-bound putrescine occurred at the same time as the onset of metastasis (Hand *et al.*, 1988). Other studies have also noted an inverse correlation between tTGase activity and the metastatic potential of clonal cell lines (Delcros *et al.*, 1986, Knight *et al.*, 1991, Beninati *et al.*, 1993). The reduction of tTGase activity has been postulated to assist tumour escape by a reduction of extracellular tTGase helping in the destabilisation of the ECM. This would stop ECM remodelling by tTGase, leaving the matrix metalloproteases to destabilise it (Hager *et al.*, 1997). This would in turn help tumour cells become detached from the primary tumour mass. A reduction of tTGase has also been shown to assist cell migration (Balklava *et al.*, 2002).

In order to clarify the role of tTGase in tumour progression, Johnson *et al.*, (Johnson *et al.*, 1994) sought to overexpress the enzyme in a malignant hamster fibrosarcoma cell line to examine the kinetics of tumour formation when those cells were reintroduced into the animal. Following transfection of tTGases cDNA into the Met B cell line, they reported that cell lines expressing high amounts of the enzyme exhibited a reduced incidence of primary tumour formation, which was accompanied by an increase in cell adhesion on FN on tissue culture plastic. No differences were noted in the *in vitro* growth rates and cell morphology between tTGase transfected cells and their sham-transfected counterparts.

A correlation between tTGase activity and tumour metastasis was proposed by (Kong and Korthuis, 1997). Using free-floating melanoma cells in denuded isolated arterioles, they reported that tTGase stabilised cell contact points with the subendothelial matrix. This is consistent with a role for cell-surface tTGase discussed earlier, and could explain how malignant cells are able to colonise other tissue types. Van Groningen *et al.*, (van_Groningen *et al.*, 1995) also noted that tTGase expression is elevated in highly metastatic human melanoma cell lines compared to weakly metastatic ones. However, Keogh *et al.*, (Keogh *et al.*, 1993) using various pharmacological agents to increase tTGase activity in human colorectal carcinoma cells concluded that the invasive behaviour of these cells was not connected to their tTGase content.

Considering the conflicting evidence, it is difficult to delineate a role for tTGase in the development of the malignant phenotype. Some have proposed that tTGase is not a tumour related marker (Takaku *et al.*, 1995) and it is clear that some malignant cell lines do express high levels of the enzyme (Mehta, 1994, Denk *et al.*, 1984, Takaku *et al.*, 1995) which suggests that the original theory of low tTGase activity in neoplastic cells is not true in all cases.

It is also worth noting that tTGase has been implicated in the body's natural defence against tumour formation (Hettasch *et al.*, 1996). This group of researchers noticed increased tTGase mRNA and gene expression in the tissue surrounding the tumour, suggesting that tTGase was helping to provide a barrier to prevent further tumour growth.

1.7 Aims of the project

The aims of this project were:

- To further clarify the role of tTGase in tumour progression with particular emphasis on its possible role in cell death using the metastatic Met B hamster fibrosarcoma as a tumour model.

- To further characterise the progression of malignancy in the P8 rat osteosarcoma, to elicit a function of tTGase in tumour progression with emphasis on its role at the cell surface and in the extracellular matrix.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 General chemicals

Most general chemicals and cell culture products were purchased from the Sigma-Aldrich Chemical Company (Poole, Dorset, UK). Otherwise, chemicals were obtained from the following suppliers:

Bio-Rad Laboratories Ltd, Watford, Hertfordshire, UK:

N, N'- Methylene bisacrylamide

BDH (Merck), Poole, Dorset:

Acetic acid, acrylamide, N, N'-dimethylcasein, glycine, hydrochloric acid, calcium chloride, dimethyl sulphoxide, D-glucose, hydrogen peroxide, sodium dodecyl sulphate, sodium acetate and sodium hydroxide.

Dako Ltd., High Wycombe, Buckinghamshire, UK:

Hydrophobic pen

Jansen Chemica, Hyde, Cheshire, UK:

Trichloroacetic acid

Life Technologies Ltd., Paisley, UK:

Geneticin (G418 sulphate)

LKB, Milton Keynes, Bucks, UK:

Optiphase Hi-safe scintillation fluid

Molecular Probes, Oregon, USA:

Biotin cadaverine and fluorescein cadaverine

Vector Laboratories Inc, Peterborough, UK:

Vector-Shield fluorescence mounting medium

2.1.2 Radiochemicals

Amersham Pharmacia Biotech, Buckinghamshire, UK:

[1, 4 ^{14}C]-Putrescine (specific activity 100-124 mCi/mmol) for use in tTGase activity determinations

2.1.3 Immunochemicals

Neomarkers, Fremont, USA:

CUB7402 anti-tTGase monoclonal anibody

Dako Ltd., High Wycombe, Buckinghamshire, UK:

Anti-mouse IgG-FITC conjugate

Calbiochem, Nottingham, UK:

Anti-mouse IgG-HRP conjugate

2.1.4 Western blotting reagents

SDS-PAGE separated proteins were transferred to nitrocellulose membranes (Gelman Biosciences, Northhampton, UK.) using an LKB semi-dry blotter. Western blots were revealed on Kodak X-OMAT film using enhanced chemiluminescence (ECL, Amersham, Little Chalfont, Bucks, UK.).

2.1.5 Protein reagents

Molecular weight markers for protein electrophoresis were supplied by Bio-Rad (Rainbow markers) and Amersham (Biotinylated markers for ECL). Marvel dried milk powder was purchased from domestic supply outlets.

2.1.6 Expression vectors

The pSG5 constitutive expression vector and neomycin resistance plasmid pSVneo were kind gifts from P. J. A. Davies (University of Texas Health Centre, USA).

2.1.7 Molecular biology kits and reagents

Plasmid DNA was purified using the Qiagen midi prep (Qiagen, Dorking, Surrey, UK) and was verified by digestion with ECoRI (Promega Ltd., Chilworth Research Center, Southampton, UK). Electrophoretically separated RNA was transferred to nylon membranes that were supplied by Bio-Rad (Zeta probe). DOTAP transfection reagent was supplied by Boeringher Mannheim (Paisley, UK)

2.1.8 Bacterial and mammalian cell lines

Plasmid DNA was amplified using the *JM109* strain of *Escherichia coli.* Met B hamster fibrosarcoma was supplied by R. C. Rees (Nottingham Trent University, UK). The P8 transplantable rat osteosarcoma was produced at Nottingham Trent University by R. N. Barnes.

2.1.9 Animals

AS rats were bred at the Department of Life Sciences, Nottingham Trent University. Golden Syrian Hamsters were obtained from Harlan, Shaw's Farm, Blackthorn, Bicester, UK.

2.1.9.1 Anaesthetics

Halothane gas anaesthetic and the Hypnorm and Hypnoval injectable anaesthetics were obtained through The Department of Life Sciences, Nottingham Trent University named Vet.

Halothane gas delivery system was purchased from IMS, Congleton, Cheshire, UK.

2.1.10 Other consumables

All water was deionised using an Elgastat system 2 water purifier (ELGA Ltd., High Wycombe, UK) or Milli Q water purifier (Millipore/Waters, Watford, UK). General laboratory consumables were obtained from the following suppliers:

BDH, Poole, Dorset, UK:

Superfrost Gold, coated glass microscope slides

Canberra-Packard, Pangborne, UK:

Scintillation vials

Corning/Bibby-Sterilin, Stone, Staffs, UK:

Tissue culture petri dishes and flasks

Sarstedt Ltd., Leicester, UK:

1.5 ml microcentrifuge tubes, 5 ml scintillation vial inserts, 15 and 50 ml sterile centrifuge tubes, 10 ml sterile pipettes and 1 ml and 200 µl pipette tips.

Sartorius Ltd., Epsom, UK:

Disposable filters (0.22 µm pore size)

Whatman Ltd, Maidstone, UK:

Whatmann 1 and Whatmann 3 MM chromatography paper

2.1.11 Equipment

General laboratory instruments were purchased from the following suppliers:

Anachem, Bedfordshire, UK:

Gilson 1 ml, 0.2 ml and 20 µl pipettes

Beckman, High Wycombe, UK:

DU-70 spectrophotometer

Bibby Sterlin, Staffordshire, UK:

Automatic pipette filler

Bio-Rad Laboratories Ltd, Watford, Hertfordshire, UK:

Mini-protean electrophoresis system, DNA-Sub electrophoresis system and 500/200 power supply.

Canberra-Packard, Pangborne, UK:

Scintillation counter (Tri-Carb 300)

Corning, Stone, Staffs, UK:

pH meter 130, spectrophotometers

Flow Laboratories, High Wycombe, UK:

MCC 340 ELISA plate reader, laminar flow cabinets

Grant Instruments, Cambridge, UK:

Water baths

Jouan Ltd., Derbyshire, UK:

Tissue culture CO_2 incubators

Leica Lasertechnik, Heidelberg, Germany:

TCSNT confocal laser microscope

MSE, Loughborough, UK:

Soniprep sonicator, microcentaur microcentrifuge and Chilspin refrigerated centrifuge

Olympus Optical Company (UK) Ltd., Middlesex, UK:

CK2 light inverted microscope, OM4 Ti 35mm camera, DP10 microscope digital camera

Pharmacia, Milton Keynes, UK:

Electrophoresis power supply, Multiphor dry blotter

Thermo-Shandon, Basingstoke, UK:

Shandon Hypercenter II and Tissue-Tek II tissue embedding center.

Carl Zeiss, Germany:

Axioskop epifluorescence microscope

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 General Cell Culture

Cell lines were grown in a humidified atmosphere at 37°C, 5%(v/v) CO_2, 95%(v/v) air. Met B and P8 cells were grown in DMEM (Dulbecco's modified essential medium) supplemented with 10%(v/v) heat inactivated FBS (foetal bovine serum), 2mM glutamine, 100 units ml^{-1} penicillin, 100µg ml^{-1} streptomycin and 1% NEAA (Non-Essential Amino Acids).

Cells were passaged and/or harvested when cells were 80-90% confluent. Cells were washed in serum-free media, prior to the addition of 1.5ml (for T75cm^3 flask) or 2.5ml (for T150cm^3 flask) of trypsin/EDTA solution (0.2% (w/v) trypsin; 100mM EDTA). The flasks were incubated at 37°C for 5 minutes. Flasks were tapped gently to ensure all cells were removed. 5ml of warm serum-containing media was added to the flasks to inactivate the trypsin. The cell suspension was transferred to a sterile centrifuge tube and spun at 1500rpm (400 x g) in a MSE bench top centrifuge. The supernatant was discarded and the cells resuspended in fresh media. Pipetting the cells up and down the pipette helps separate the cells in the suspension. A 10µl sample was counted using the Trypan blue method (described below) and the number of cells in the suspension calculated. A suitable number of cells were seeded ($4x10^5$ for a T75cm^3) into a new flask and 10-15ml of media added to the cells. Cells were then incubated as described above.

2.2.1.2 Assessment of the number of viable cells by exclusion of trypan blue

Cells were harvested as described above. 10µl of cell suspension was added to 10µl of Trypan blue solution (Sigma) and incubated at 37°C for 5 minutes. Cells were counted on a haemocytometer (blue cells are non-viable, clear cells are viable) and the number of viable cells in the original cell suspension

calculated (remembering to take into account the 1:1 dilution in Trypan blue solution).

2.2.1.3 Freezing Down Cells

Cells were harvested and spun down at 1500rpm (400 x g) for 5 minutes. The supernatant was removed and the pellet resuspended in cold freezing mixture (5% DMSO (v/v) in foetal bovine serum) to give a final concentration of 10^7 cells per ml. The suspension was transferred into 1ml cryovials and placed at –70°C overnight before being stored in liquid nitrogen.

2.2.1.4 Thawing Out Frozen Cells

Cells were removed from storage and placed in a 37°C water-bath until thawed. Immediately after thawing, the cells were transferred into a sterile centrifuge tube and diluted in 5ml of warm media. Cells were then centrifuged at 1500rpm (400 x g) for 5 minutes. The supernatant was removed and this wash step repeated. Cells were resuspended in media, plated out into a new T75cm^3 flask and incubated as described above.

2.2.1.5 Cell Homogenisation

Cells were harvested and spun down into a pellet. Cells were then resuspended in homogenisation buffer (0.25M sucrose, 5mM Tris-HCl (pH7.4), 2mM EDTA, containing protease inhibitors PMSF (20µg/ml), leupeptin (10µg/ml), pepstain (1µg/ml), benzamidine (780µg/ml). Cells were then sonicated on ice for 3x5 seconds with 15 second cooling intervals.

2.2.1.6 Coating wells with ECM proteins

Solutions of fibronectin were made up to 5µg/ml in PBS. 50µl of fibronectin solution was transferred into each well and left at 4°C overnight. Coated surfaces were blocked with 3% (w/v) BSA in Tris-HCl buffer for one hour at room temperature and washed three times with SFM prior to the addition of cells.

2.2.2 Measurement of tTGase Activity

2.2.2.1 Measurement of tTGase activity by incorporation of [^{14}C]-putrescine into N,N'-dimethylcasein

The principle of the assay relies on the ability of the enzyme to catalyse the incorporation of a radioactively labelled primary amine into a protein acceptor substrate (Lorand, 1972).

Reactions were carried out at 37°C in 1.5ml Eppendorf tubes. Each tube contained 10µl 50mM Tris-HCl (pH7.4), 10µl 38.5mM DTT, 10µl 12mM [1,4-^{14}C]-putrescine stock*, 20µl 25mg/ml N,N'-dimethylcasein in 50mM Tris and either 5µl 50mM $CaCl_2$ or 5µl 50mM EDTA. The reaction is started by the addition of 45µl of sample. At 10 and 20 minute intervals, 3 x 10µl aliquots were removed from each tube and spotted onto 10mm^2 filter paper squares and immediately placed into ice-cold 10% (w/v) TCA for at least 10 minutes. A set of filter papers without sample were also washed as a reaction blank. The filter papers were then washed for 5 minutes in each of the following:

- 3 times in ice-cold 5% (w/v) TCA
- 1:1 acetone:ethanol
- acetone

At the end of the assay, 10µl aliquots of random samples were spotted onto filter paper squares and left to dry. These were used as a measurement of total counts in order to estimate counting efficiency. The filter papers were left to dry overnight. Once dry, all filter papers were placed in scintillation tubes, to which 2ml Optiphase High Safe liquid scintillation fluid was added and the tubes counted in a Packard Liquid Scintillator.

*12mM [1,4-^{14}C]-putrescine stock:

Add 50µl 243mM cold putrescine to 1ml of [1,4-^{14}C]-putrescine (250µCi./ml), giving a working stock of 12mM putrescine with a specific activity of 3.97mCi/mmol.

2.2.2.2 In situ tTGase activity

The determination of *in situ* transglutaminase activity was as previously described by (Verderio *et al.*, 1998). All solutions prior to fixation were supplemented with the protease inhibitors 1mM leupeptin, 1mM benzamidine, 1mM pepstatin and 1 mM PMSF. Unfixed cryostat sections were washed twice with PBS (pH7.4) prior to incubation with 100µl of 0.5mM fluorescein-cadaverine (Molecular Probes, Cambridge Bioscience, UK) and 2.5mM $CaCl_2$ in 10mM Tris/HCL pH 7.4 for 1 hr at 37°C. As stated previously the use of unfixed sections dictates that only insoluble tTGase is detected and soluble enzyme is lost during rehydration. Negative controls included the replacement of $CaCl_2$ with 2mM EDTA. After washing three times with PBS (pH7.4) the sections were fixed with –20°C methanol for 10 min. After washing three times in PBS (pH7.4), the sections were with 5µg/ml propidium iodide (PI). Sections were washed again to remove excess PI and mounted with Vector-Shield fluorescent mounting media (Vector Laboratories, Peterborough, UK).

Sections were visualised using a Leica TCS NT confocal microscope (Leica). Settings were varied to obtain optimum picture quality.

2.2.2.3 Measurement of cell surface tTGase activity by biotin-cadaverine incorporation into fibronectin

Transglutaminase activity associated with the cell surface was measured by the incorporation of biotin-cadaverine into FN (Jones *et al.*, 1997). Cells were seeded into 96-well plates (2x 10^4 cells/ well) precoated with 5 µg/ml FN in 100 µl serum free DMEM containing 0.132 mM biotin-cadaverine. Positive and negative control samples were also included, which consisted of 100

ng/well gplTGase that was incubated in a reaction mixture consisting of 0.1 M Tris buffer pH 7.4, 10 mM DTT, 0.132 mM biotin-cadaverine and either 10 mM $CaCl_2$ or 10 mM EDTA. Cells and enzyme were incubated for 1 hour at 37°C and then washed twice with PBS pH 7.4 containing 3 mM EDTA to stop incorporation. A solution of 0.1% (w/v) deoxycholate in PBS (100 µl) was then added to each well, incubated at 37°C with gentle shaking for 10 minutes to remove the cells but retaining the extracellular matrix. The remaining FN layer was washed three times with Tris-HCl pH 7.4, and the wells were then blocked with 3% (w/v) BSA in Tris-HCl for 30 minutes at 37°C. Biotin-cadaverine incorporation into FN was detected with an Extravidin peroxidase conjugate diluted 1/5000 in blocking buffer, which was incubated for 1 hour at 37°C and revealed using tetramethylbenzidine (TMB) as the substrate. The reaction was stopped by the addition of 2.5N H_2SO_4 and the resultant colour was read in an ELISA plate reader at 450nm.

2.2.2.4 Detection of cell surface/ECM tTGase by ELISA

This is a modification of a normal ELISA technique based upon recognition of a specific antigen by immuno-cytochemistry. Instead of working on either cell homogenates or cell fractions, the assay uses cells, which have been grown on the plates. Cells were seeded at a concentration of 1.5×10^4 cells/well in a 96-well plate and grown overnight before labelling. The primary antibody Cub7402 (Neomarkers) was diluted 1 in 1000 in cell growth medium and 100 µl added to each well and left for 2 hours. Following incubation, medium was removed, and the cells were washed three times with PBS (pH 7.4) and then blocked with blocking solution (5% (w/v) fat-free dried milk in PBS, pH 7.4) for 30 minutes. The plate was washed three times with PBS, pH 7.4 and both cells and antibody then fixed with 3.7%(w/v) paraformaldehyde in PBS, pH 7.4 for 15 minutes at room temperature. After fixation, another series of washes with PBS, pH 7.4 were performed and the secondary antibody (mouse IgG-HRP) diluted 1 in 1000 in blocking buffer

was added to each well (100 µl/well) and incubated for 2 hours at room temperature followed by three washes with PBS, pH 7.4 as before.

Development of the assay was performed using phosphate-citrate buffer with urea hydrogen peroxide and 7.5%(w/v) TMB as described above (section 2.2.14). The reaction was stopped by addition of 50 µl of 2.5 M sulphuric acid and the absorbance read at 450 nm using a Spectrafluor 96 well plate reader.

For normalisation of the assay, identical cell numbers were grown in 96-well plate in parallel and solubilised in 0.1%(w/v) deoxycholate/2 mM EDTA as described in section 2.2.6.2. Proteins were precipitated in 10%(w/v) final volume TCA and assayed using the BCA method (Brown *et al.*, 1989) as described previously (section 2.2.6.2). The measured tTGase protein was then expressed as absorbance value at 450nm per 1.0 mg of deoxycholate-soluble protein.

2.2.3 Analysis of proteins

2.2.3.1 Protein determination

The total protein content in cell homogenates was determined by the Lowry method (Lowry *et al.*, 1951). When using buffers containing a high percentage of SDS or other detergents, the Bicinchoninic acid assay (BCA) was used (Brown *et al.*, 1989).

2.2.3.2 Lowry Method

The commercial kit from Bio-Rad based on the Lowry method (Lowry *et al.*, 1951) was used following the manufacturer's instructions. Briefly, 5µl sample, 25µl of reagent A and 200µl of reagent B were added to the 96 well plate, left to incubate for 10 minutes before reading the absorbance at 750nm using a SpectraFluor 96-well plate reader. BSA solutions ranging from 1-5mg/ml were used as protein standards to produce the calibration graph.

2.2.3.3 Bicinchoninic acid (BCA) Method

Cells were solubilised by the addition of 0.1%(w/v) sodium deoxycholate containing 2 mM EDTA with gentle shaking for 10 minutes at room temperature. Proteins from the cell lysate were precipitated by the addition of 50%(w/v) trichloroacetic acid (TCA) to a final concentration of 10%(w/v) followed by incubation for 30 minutes on ice. Proteins were pelleted by centrifugation at 13,000 × g for 10 minutes. The supernatant was removed and the proteins were re-suspended in 1/10 of the initial volume 0.1M NaOH containing 5%(w/v) SDS. 5µl of protein suspension were mixed with 100µl of BCA standard working reagent in a 96-well plate and incubated at 37°C for 30 minutes. The absorbance of samples was then read at 570nm using a SpectraFluor 96 well plate reader. BSA solutions ranging from 0.2-1mg/ml were used as protein standards to produce the calibration graph.

BCA working reagent was obtained by addition of 25 parts of solution A (1%(w/v) bicinchoninic acid (sodium salt), 2%(w/v) sodium carbonate, 0.16%(w/v) sodium tartrate, 0.4%(w/v) sodium hydroxide, 0.95%(w/v) sodium hydrogen carbonate, pH11.25) to 1 part of solution B (4%(w/v) copper sulphate).

2.2.3.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The method employed was a modification of that described by Laemmlli (1970) for use with vertical slab gel apparatus. Gels were cast in Bio-Rad Mini Protean II gel systems and consisted of a 3% (w/v) polyacrylamide stacking gel and a 10% (w/v) resolving gel. Stacking gels were made using a Tris-SDS stock solution, pH 6.8 (0.25 M Tris, 0.2% (w/v) SDS) and resolving gels contained a Tris-SDS solution pH 8.8 (0.75 M Tris. 0.2% SDS). The acrylamide stock solution used for all gels consisted of 30% (w/v) acrylamide and 0.8% (w/v) N, N'-methlylene bisacrylamide (10% gels were made of 5ml of 30% (w/v) acrylamide and 0.8% (w/v) N, N'-methlylene bisacrylamide,

3.75ml Tris-SDS pH8.8, 6.25ml water, 50µl 10%(w/v) $(NH_4)_2SO_4$ and 10µl TEMED).

Prior to casting, electrophoresis plates were polished with Repel-Silane to facilitate the removal of gels following electrophoresis. Electrophoresis equipment was assembled as directed by the manufacturers, and glass plates were separated by 0.75 mm spacers.

Gel reagents were added to a Buchner flask in the order of the recipe described above. Prior to the addition of ammonium persulphate and TEMED, the gel was degassed using a vacuum pump for 5 minutes. The freshly prepared 10% (w/v) ammonium persulphate and N, N, N, N′-TEMED was then added to the gel, and the solution was pipetted between the glass plates. Water-saturated butan-2-ol was poured on the top of the gel to an approximate depth of 5 mm to provide the gel with a flat upper surface, and the gel was allowed to polymerise for 1 hour at room temperature.

The upper surface of polymerised resolving gels were washed three times with Tris/SDS pH 8.8 solution to remove the butan-2-ol, and the edge of the gel was gently blotted dry using filter paper. Stacking gels were prepared by combining 0.65 ml of acrylamide solution, 1.25 ml of Tris/SDS stock pH 8.8 and 3.05 ml of water. The solution was degassed, and polymerisation was initiated by the addition of 25µl 10% (w/v) ammonium persulphate and 5 µl of TEMED. The gel was quickly pipetted between the glass plates and the comb that forms the sample wells. Any trapped bubbles were removed by gently tapping the gel. Polymerisation was allowed to proceed for one hour at room temperature.

The sample well comb was gently removed from polymerised stacking gels and wells were washed and filled with Tris-glycine electrode running buffer pH 8.5 (0.025 M Tris, 0.192 M glycine and 0.1% (w/v) SDS). Samples were equalised for protein concentration, combined with the appropriate volume

of 2X strength Laemmli loading buffer (125 mM Tris-HCl, 20% (v/v) glycerol, 4% (w/v) SDS, 2% (v/v) mercaptoethanol and 0.02 mg/ml bromophenol blue) and pipetted into the sample wells using a Hamilton syringe. Electrophoresis was performed at 100V for approximately 2 hours until the bromophenol blue marker dye was approximately 5 mm from the bottom of the gel.

2.2.3.5 Western blotting of SDS-PAGE separated proteins

Electrophoretic transfer of SDS-PAGE separated proteins to nitrocellulose was performed as described by Towbin *et al.*, (1986) using a LKB semi-dry blot system. Briefly, the graphite anode was wetted with distilled water and 9 sheets of filter paper cut slightly larger than the polyacrylamide gel were soaked in continuous transfer buffer (48.8 mM Tris, 39 mM glycine, 0.0375% (w/v) SDS and 20% (v/v) methanol) and placed on the electrode. A nitrocellulose sheet was wetted in transfer buffer and placed on top of the filter papers, ensuring that all air bubbles were expelled. The gel was then detached from the electrophoresis plates in transfer buffer and applied to the nitrocellulose membrane. Nine further wetted filter paper sheets were placed on top of the gel and gentle, even pressure was applied to remove any trapped air pockets. The graphite cathode was then connected to the apparatus and placed on top of the filter paper assembly. Western blotting was performed for 45 minutes at 45 mA per gel.

Once 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 a Ponceau solution (0.2% (w/v) Ponceau S, 0.4% (v/v) glacial acetic acid) and the stain was removed using PBS-Tween. Washed blots were blocked by incubation in a solution containing 5% (w/v) dried milk powder in TBS-Tween before being immunoprobed with the appropriate primary antibody.

2.2.3.6 Immunoprobing of Western blots with an anti-tTGase monoclonal antibody

For optimal detection of tTGase antigen by immunoblotting with the anti-tTGase monoclonal antibody Cub7402, 15µg total cellular protein were loaded onto SDS-PAGE gels. Blots were blocked overnight at 4°C in TBS-Tween/Marvel, then incubated with Cub7402 diluted 1/1000 in the same buffer for 2 hours at room temperature with gentle shaking. Once completed, the nitrocellulose was washed 3 times for 5 minutes with TBS-Tween/Marvel and transferred to a new plastic tray. Blots were then incubated with an anti-mouse IgG horseradish peroxidase (HRP) conjugate diluted 1/1000 in TBS-Tween/Marvel for 1 hour 30 minutes at room temperature with gentle shaking. Western blots were visualised using enhanced chemiluminescence (ECL: Amersham). Blots were retained in their plastic trays and thoroughly washed with PBS containing 0.05% (v/v) Tween 20 to remove the Marvel milk. Blots were washed again in PBS alone to remove traces of Tween, and ECL reagents A and B were mixed in a 1:1 ratio in a total volume of 4 ml. The ECL mixture was poured onto the nitrocellulose, and was shaken over the surface of the blot for one minute. Once completed, the blot was removed from the plastic tray using forceps and excess reagent was wiped from the nitrocellulose. Treated blots were covered with cling film, ensuring that no creases or bubbles were present, and were placed face-up in an X-ray cassette and secured by masking tape. In a dark room, a piece of X-ray film was taped over the wrapped blot and the cassette was closed for the optimum time period. The optimum exposure time varied for each individual blot and ranged from 10 seconds to 20 minutes. Exposed blots were washed in developer for one minute (Kodak, LX-24, diluted 1/5 with water), immersed in fixative (Kodak, FX-40, diluted 1/5 with water) and rinsed in distilled water.

2.2.4 Cell biology techniques

2.2.4.1 Isolation of SDS-insoluble material

2.2.4.1.1 From cells

SDS-insoluble material was isolated by the method of (Knight *et al.*, 1991). Cells were harvested by trypsinisation. Cells in the culture media were also collected by centrifugation and pooled with trypisinised cells. Cells were resuspended in serum-free media at a concentration of $2x10^8$ cells/ml. 500µl of single-cell suspension was aliquoted and 50µl of 20%(w/v) SDS and 50µl of 0.1%(w/v) dithiothreitol (DTI) were added to it. This mixture was then boiled for 5 min. After cooling, the SDS-insoluble material was washed in 0.1%(w/v) SDS in PBS, then finally resuspended in PBS.

2.2.4.1.2 From tissue

Tissue was harvested as described in Section 2.2.3.5. The tissue was immediately weighed and measured before being placed onto a hard wax disc and a few drops of homogenising buffer (142mM NaCl, 66mM KCl and 5mM EDTA in Dulbecco's PBS pH7.4) and finely chopped with a M^{c}Ilwain tissue chopper. The tissue was then chopped again at 90° to the original cutting. The chopped tissue was then digested with collagenase type VII (Sigma) at 500 units/ml in homogenising buffer (described above). The samples were incubated for 3 hours at 37°C in a shaking water bath. After the incubation, 10ml SDS (5mg/ml) and 5ml DTT (6.25mg/ml) were added to the samples and boiled for 5 min. All samples were allowed to cool before being centrifuged at 750 x g for 5 min. The supernatant and any fibrin, which appears as a black precipitate on top of the white pellet of insoluble polymer was removed. This occasionally required further centrifugation. The polymer was cleaned by washing in 0.1% SDS (w/v) in PBS, before finally being resuspended in PBS. The 20µl of SDS-insoluble material was then air-dried onto a slide and stained for DNA fragmentation.

2.2.4.2 Determination of tTGase action following cell damage

Cells were removed from culture by trypsinisation, washed in serum-free media and immediately incubated with 0.5mM fluorescein-cadaverine in complete growth media for 45 min at 37°C. The cells were collected by centrifugation, air-dried onto a glass slide and fixed in Methanol (-20°C) for 15 min. Slides were gently washed in PBS, mounted in Vector-Sheild and viewed by fluorescence microscopy (Zeiss Axioskop) using filters suitable for fluorescein. Photographic data was then analysed using the Aequitas image analysis computer software program.

Envelope formation as mediated by tTGase was visualised by treating the cells after incubation with fluorescein-cadaverine.

2.2.4.3 Fluorescein-Cadaverine incorporation into the cell surface

To investigate the activity of cell surface tTGase, a method of (Verderio *et al.*, 1998) was employed. Cells ($2x10^5$) were harvested and resuspended in 200µl of media containing 50µM FITC-Cadaverine. The cells were incubated in 15ml plastic tubes for 2 or 4 hours at 37°C, 5% CO_2. Tubes were gently shaken every 20 min to prevent the cells from attaching to the tubes. After the incubation, the reaction was stopped by the addition of 5mM EDTA in PBS. The cells were pelleted at 1500rpm (400 x g) for 5 minutes. The cells were washed twice in 5mM EDTA in PBS and air-dried onto a slide. The cells were fixed in –20°C methanol for 15 min followed by washing once in –20°C methanol and three times in PBS before counter-staining with propidium-iodide (5µg/ml). Cells were washed and mounted in VectorShield, before a coverslip was sealed onto the slide and the cells viewed under a Leica confocal microscope.

2.2.4.4 Fluorescein-Cadaverine incorporation into the ECM

Cells were harvested and counted as described in Section 2.2.1. Cells ($2x10^5$) were seeded into a chamber of a chamber slide in complete media containing 50µM FITC-Cadaverine. The chamber slide was incubated at 37°C, 5% CO_2

for 24 hours, thus allowing cells to attach and lay down some extracellular matrix. The supernatant was removed and the cells fixed in -20°C methanol for 15 min. The cells were washed in –20°C methanol to remove excess FITC-Cadaverine. The cells were washed three times in PBS and counter-stained with propidium-iodide (5μg/ml for 15 min) and washed three times with PBS. The slides were mounted in VectorShield and a coverslip applied before being viewed using a Leica confocal microscope.

2.2.4.5 Cell attachment assay

Cells were trypsinized and 10^4 cells seeded into each well of a 96 well plate (the wells were sometimes coated with an extracellular matrix (ECM) protein such as fibronectin –see above). The plate was incubated at 37°C, 5%(v/v) CO_2 for 2 hours. The wells were washed very gently with PBS (pH7.4). Cells were then stained with 100μl crystal violet (0.5%(w/v) in 70%(v/v) ethanol) per well and incubated at room temperature for 15 minutes. The crystal violet was removed and the cells washed in PBS. The cells were observed under an inverted microscope and photographed. The cells were solubilized in 30% (v/v) acetic acid and absorbance read in a plate reader at 540nm.

2.2.4.6 Antibody inhibition of cell attachment

Cells were trypsinized and 10^4 cells resuspended into 100μl of media. Control cells were also collected. CUB7402 α-tTGase mAb was added to the cells to give a final concentration of 100μg/ml. GIB1 α-wheat gliadin mAb was added to the control cells. All cells were incubated at 37°C for 2 hours before being assayed in the cell attachment assay described above.

2.2.5 Apoptosis detection

2.2.5.1 ATP:ADP ratio

The ApoGlow kit (Lumitech) measures the levels of ATP and ADP in cells. From this, it can be determined the state of the cell (i.e. whether the cell is proliferating or undergoing necrosis or apoptosis). Cells (10^5) were grown in

white-walled, clear-bottomed 96-well plates, in media with or without 1μM dexamethasone for 48 hours. After 24 hours, any apoptotic inducing agent was added and left for the remaining 24 hours. ATP:ADP ratios were then calculated using the ApoGlow kit (Lumitech) and was carried out as per manufacturers instructions. Percentage viabilities compared to normal, untreated Met B cells were also calculated.

2.2.5.2 Induction of Caspase-3

The ApoAlert kit (Clontech) measures the activity of the Caspase-3 apoptotic enzyme by the cleavage of a specific substrate, conjugated to pNa, which when cleaved produces a colour. This can be read on a spectrophotometer. Cells (10^5) were seeded into a 96-well plate and grown in media with or without 1μM dexamethasone for 48 hours. After 24 hours, any apoptotic inducing agent was added and left for the remaining 24 hours. The activity of Caspase-3 was monitored using the ApoAlert kit (Clontech) and was carried out as per manufacturers instructions. The higher the absorbance (450nm), the higher the activity of Caspase-3 in the sample

2.2.5.3 DNA fragmentation

DNA fragmentation kits work by labelling the Klenow fragments produced during the apoptotic pathway. These are then detected by an antibody conjugate to the label, giving a visual display of apoptosis.

2.2.5.3.1 ApopTag (Oncor-Appligene)

Cells (10^6) were seeded into a petri-dish containing a glass slide and grown in media with or without 1μM dexamethasone for 48 hours. After 24 hours, any apoptotic inducing agent was added and left for the remaining 24 hours. DNA fragmentation in cells was detected using the ApopTag kit (Oncor-Appligene) and carried out as per manufacturers instructions.

2.2.5.3.2 FraGeL (Calbiochem)

DNA fragmentation in SDS-insoluble material isolated from tissue samples (see Section 3.2.1.2) was carried out using the FraGeL kit (Calbiochem) and

carried out as per manufacturers instructions for tissue sections / cells grown on slides.

2.2.6 Molecular biology methods

2.2.6.1 Bacterial transformation

Escherichia coli JM109 were transformed using a calcium chloride transfection protocol. Cells were cultured overnight in 35 ml of Luria Broth pH 7.5 (LB; 1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract and 1% (w/v) NaCl in H_2O) and placed in an incubator/shaker at 37°C/ 250 rpm. The following day, 35 ml of fresh LB was inoculated with 1 ml of overnight culture, and cells were allowed to grow exponentially until the culture reached an absorbance value of 0.3-0.5 at a wavelength of 600 nm. Cells achieving a suitable growth state were chilled in an ice bucket for twenty minutes and centrifuged at 3000 rpm for 15 minutes at 4°C. The supernatant was discarded from the cell pellet and the bacteria were resuspended in 20 ml of ice cold 100 mM $CaCl_2$ and mixed gently. The cells were again sedimented by centrifugation and the washing procedure repeated twice, and the cell pellet was resuspended in 1 ml of ice cold 100 mM $CaCl_2$ following the final wash. Cells were maintained in chilled $CaCl_2$ for 1 hour, and then divided into 200 µl aliquots in sterile microcentrifuge tubes prior to the addition of plasmid DNA. *JM109* cells were transformed with pSG5 and pSVneo DNA plasmids. Each plasmid used for bacterial transformation contained an ampicillin-resistance sequence downstream of a bacterial promoter.

JM109 cells were chilled in the presence of DNA for 30 minutes, subjected to a 90 second heat shock at 42°C, then briefly placed on ice following the addition of 800 µl of LB. Recovering cells were incubated for 40 minutes at 37°C with gentle mixing and 200 µl of the cell suspension was used to inoculate LB agar plates containing ampicillin. Plates were incubated overnight at 37°C, and the following day a single colony was isolated and grown to provide the stock supplies of transformed cells.

2.2.6.2 Plasmid preparation

Transformed *JM109* cells were grown in 250 ml LB as described in the previous section. Plasmid DNA was extracted from centrifuged cells using a Qiagen plasmid midi kit according to the manufacturer's protocol. Ethanol washed DNA pellets were resuspended in 200 µl of nuclease-free water and analysed by UV spectrophotometry and restriction enzyme digestion/agarose gel electrophoresis.

2.2.6.3 Determination of DNA and RNA concentration and purity by UV spectrophotometry

The concentrations of DNA and RNA were determined by their absorbance at 260 nm following dilution in endonuclease-free water. Quartz cuvettes were washed with ethanol and endonuclease-free water prior to assaying. The concentration of DNA and RNA were calculated using the following calculations:

DNA concentration (µg/ml) = A_{280} nm x 50 x dilution factor

RNA concentration (µg/ml) = A_{280} nm x 40 x dilution factor

Typically, DNA was diluted 1/100 and RNA was diluted 1/1000 for concentration determinations.

DNA and RNA purity was assessed by the ratio of A_{260} nm to A_{280} nm absorbance values. A nucleic acid solution with an A_{260}/A_{280} ratio of between 1.5-2.0 was deemed acceptable for use in most molecular biology protocols.

2.2.6.4 Cell transfection

For stable transfections, $0.2\text{-}0.5\times10^6$ cells were seeded into a 10cm tissue culture petri dishes one day prior to the DNA transfer. Transfection of the P8 osteosarcoma cell line with pSG5TG was achieved by cotransfecting cells with 10 µg of plasmid vector and selection vector pSVneo (plasmid ratio 9:1,

respectively) using the liposome-based transfection reagent DOTAP (Boeringher Manheim) following manufacturer's protocol. Clones were obtained by growing the cells in normal media supplemented with 400μg/ml of G418 antibiotic. This kills off cells not containing the pSVneo plasmid conveying G418 resistance.

2.2.6.5 Cloning of transfected cell lines

Stably transfected cell lines were created by the separation and removal of clonal colonies present on the transfection stock plates. Petri dishes were gently washed 3 times with SFM and 3-5 μl of 0.25% (w/v) trypsin in PBS pH 7.4 was directly pipetted onto the chosen colony, ensuring that the tip of the pipette remained in contact with the expelled trypsin. The trypsin was allowed to cover the cells for approximately 30 seconds, and then drawn back into the pipette. The trypsin was then repeatedly spotted and retracted onto the colony for a further 15 seconds, before being transferred to a sterile 15 ml centrifuge tube containing 5 ml of complete medium. Another selected colony was removed in this way and the stock plate was gently covered with SFM to prevent the cells drying out. The medium containing the removed cells was vigorously passed several times through a sterile 10 ml pipette to disperse the dislodged cells, and was plated into a T25 tissue culture flask. The flask was examined using an inverted light microscope to ensure that cells had been collected and transferred to an incubator.

Before further colonies were removed from the stock plate, the dish was gently washed 5 times with SFM to remove any detached cells that were not collected with the trypsin.

2.2.6.6 Isolation of RNA

To prevent ribonuclease contamination of RNA samples, all non-disposable plasticware was treated with 0.1% (v/v) DEPC in deionised water and autoclaved. Disposable, sterile plastic consumables were used wherever

possible and all glassware was rinsed with chloroform. All water used for RNA work was treated with 0.1% (v/v) DEPC and autoclaved prior to use.

Total cellular RNA was extracted from confluent cells in 10 cm petri dishes or tissue using TRI reagent according to the manufacturer's protocol. Isolated RNA was dissolved in 10 µl of RNase free water and quantified using UV spectrophotometry, as described earlier.

2.2.6.7 RNA electrophoresis and Northern blotting

RNA formaldehyde/agarose gels were prepared by melting 0.6 g of agarose in 5 ml of 10X MOPS solution pH 7 (200 mM MOPS, 50 mM sodium acetate and 10 mM EDTA, 10 mM EGTA) and 44 ml of water. Once cooled to 42°C, 2.6 ml of formaldehyde were added and the solution was gently mixed and poured into a RNA gel-casting chamber to solidify at 4 °C.

RNA samples were prepared by combining 30 µg of RNA with 25 µl of RNA sample buffer (80% (v/v) deionised formamide, 8% (v/v) formaldehyde, 50 µl 10X MOPS buffer, 0.01% (w/v) bromophenol blue) and heated at 65°C for 15 minutes. Prepared RNA was pipetted into the sample wells and electrophoresis was performed for 3 hours using 1X MOPS as tank buffer.

RNA gels were visually assessed and photographed using UV illumination to confirm the presence of the 28S and 18S ribosomal RNA subunits. RNA was transferred to nylon membranes (Zetaprobe, BioRad) using a modified method described by (Sambrook *et al.*, 1989). The Northern transfer apparatus consisted of a glass plate placed over a glass dish containing a reservoir of 20X SSC pH 7.0 (3M NaCl, 0.3M sodium citrate). A 3MM paper wick was constructed and placed on the glass plate so that the overhanging edges were completely immersed in the reservoir of transfer buffer below. The RNA gel was inverted and placed onto the wick, and areas of the wick not in contact with the gel were covered with parafilm. A nylon membrane was applied to the upper surface of the gel ensuring that no air bubbles were

trapped between the surfaces, and several sheets of 3MM paper and approximately 10 cm of paper towels were placed over the membrane. The assembly was completed by the addition of a further glass plate and a weight of approximately 500 g that was secured by a clamp, and transfer was allowed to proceed overnight. The following day, the apparatus was disassembled and the membrane was marked to identify the position of the sample wells, and the RNA was cross-linked to the filter using a Stratagene Stratalinker set to auto cross-link mode (1.2 mJ/cm^2). UV irradiated membranes were washed once in 1X SSC pH 7.0, air-dried and stored for future hybridisation.

2.2.6.8 In situ hybridisation

Sections were dewaxed in xylene and rehydrated in alcohols up to water, as in the staining of paraffin wax sections. Sections were treated with proteinase K solution (5µg/ml) at 37°C for 30 mins. After washing, the slides were fixed using pre-cooled 0.4% neutral buffered formalin to 4°C for 20 mins. The slides were washed again in DEPC water. The sections were incubated in prehybridisation buffer (196µl DEPC water, 2µl 0.5M Tris-HCl [pH 7.4], 100µl 20x Standard Saline Citrate (SSC), 2µl 0.05M EDTA, 200µl 50% Dextran sulphate, 500µl Formamide [add in a fume cupboard]) at 42°C for 1 hour. Whilst incubating, a stock solution of digoxigenin labelled cDNA probe solution was prepared (10µg/ml labelled probe, 1µl 0.5M Tris-HCL [pH 7.4], 50µl 20x SSC, 1µl 0.05M EDTA, 100µl 50% Dextran sulphate, 250µl Formamide [add in fumecupboard] and made to 500µl with DEPC-water). After the incubation, the pre-hybridisation buffer was tipped of and 20µl of stock solution was added to each section. The tissue and probe were then denatured by incubation at 95°C for 10 mins and incubated at 42°C overnight in a humidity chamber. The following morning, the sections were washed several times in SSC before being washed in wash buffer (0.1x SSC, 2mM $MgCl_2$, 0.1% Triton-X100) at 42°C for 30 mins. Sections were then blocked in blocking solution (3% bovine serum albumin (BSA) in Tris buffered saline (TBS) containing 0.1% Triton-X100) for 15 mins. Anti-

digoxigenin alkaline phosphatase conjugated antibody was then added diluted 1:600 in blocking solution for 30 mins at 37°C. Slides were then washed several times in TBS before the addition of alkaline phosphatase substrate buffer for 5 mins to equilibrate. Alkaline phosphatase substrate was then added for 30 mins at room temperature, followed by washing in running tap water. The slides were then mounted in aqueous mountant and viewed under a microscope.

2.2.7 Animal studies

2.2.7.1 Sub-cutaneous implantation of tumour tissue

Anaesthesia was induced and maintained using Halothane gas anaesthetic (IMS). Once the animal was anaesthetised, the right flank of the animal was then shaved and sterilised using 70% ethanol. A small incision was then made in the shaved area, taking care not to penetrate the muscular tissue. A subcutaneous pocket was made by inserting blunt end forceps into the incision and gently separating the connective tissue. A small piece of tumour tissue was placed into this pocket and the wound closed using a stainless steel wound clip (Clay-Adams). The animal was allowed to recover from the anaesthesia and was monitored for signs of distress throughout the experimental period.

2.2.7.2 Sub-cutaneous injection of tumour cell lines

Anaesthesia was induced using Halothane gas. Cell suspension was drawn into a syringe, ensuring all air was dispersed through the needle by tapping the syringe needle up gently until all air bubbles had floated to the surface. Air was then pushed out of the syringe and needle. The skin on the right flank of the animal was then pinched and lifted to form a cavity between the layer of skin and muscle below. Cells were injected into this cavity, taking care to inject the suspension slowly to prevent the rupturing of cells due to shear forces. Upon needle withdrawal, the puncture hole was held for 15 seconds to allow the cell suspension to disperse and not come out of the hole. The

animal was then allowed to recover from anaesthesia and was monitored for signs of distress throughout the experimental period.

2.2.7.3 Resection studies

Resection was only carried out on tumours less than 1.5cm in diameter. Anaesthesia was induced by intraperitoneal injection of a mixture of Hypnorm & Hypnoval injectable anaesthetics as directed by accompanying instructions to the anaesthetics. Taking care to ensure the animal was completely anaesthetised, the area of the tumour was shaved and sterilised using 70% ethanol. An incision into the skin was made just below the tumour using a sterile scalpel, to slightly larger than the tumour diameter. Sterile scissors were then used to cut away the connective tissue from around the tumour, taking care not to cut any cutaneous or muscular tissue. Once the tumour mass was removed from the animal, the underside of the skin and surrounding areas were examined for primary tumour left behind. Any further tumour tissue discovered was cut away to prevent tumour regrowth. Excess blood was moped up using sterile wipes and the incision sewn back together using sutures. The animal was monitored closely until consciousness was regained. Anaesthetic cream was often applied around the wound, but care was taken not to get any in the wound itself. Stitches were removed after several days, when the wound was beginning to heal. Anaesthesia was induced using Halothane gas anaesthetic and stitches carefully removed by cutting the knot and pulling the suture through the skin.

When the rat began to show signs of distress, or when the primary tumour load had re-grown significantly the rat was sacrificed and the secondary tumours harvested from primarily lung tissue.

2.2.7.4 Detection of secondary tumours without resection

The tumours were allowed to grow and were removed when the tumour reached a suitable size. To remove the tumours, the rat was anaesthetised using Halothane gas anaesthetic and blood was drained from the heart by

cardiac puncture. This was to help reduce the amount of blood in the lungs (to look for metastasis) and in the tumour itself. After maximum amount of blood was drawn from the heart, the animal was killed by dislocation of the neck. The heart and lungs were then dissected out from the body and washed in PBS. The lungs were then inflated using non-waterproof Indian ink (15%(v/v) containing 0.5%(v/v) ammonia solution) to check for metastasis (white patches in black stained lung tissue). The trachea was then ligated and the lungs fixed in Feketes solution (45%(v/v) ethanol, 20%(v/v) dH_2O, 5%(v/v) formaldehyde and 30%(v/v) glacial acetic acid). Secondary tumours detected this way can be seen in Figure 2.1.

2.2.7.5 Harvesting of primary tumour load

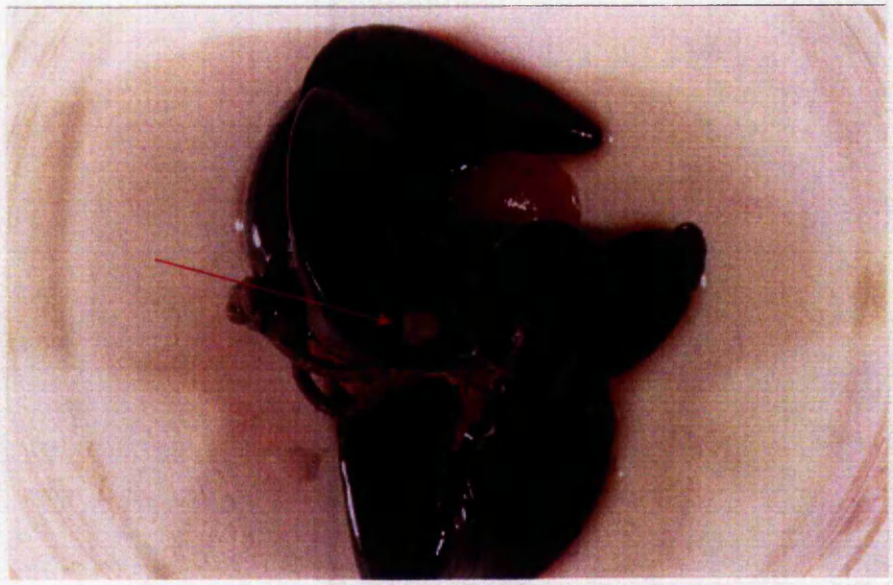
Animals were sacrificed by dislocation of the neck and tumours were removed as described above in the resection studies. The tumour mass was placed into appropriate buffer as soon as possible and placed on ice.

2.2.7.6 Induction of experimental metastases

Rats were placed into a chamber designed for taking blood pressure. The tail is wiped with methanol to help reveal the position of the tail vein. Once located the syringe is filled with cell suspension and air expelled as described above. The needle is pushed slowly into the vein while applying a negative pressure on the syringe plunger. When the syringe draws blood, the needle is in the tail vein. The plunger of the syringe is then slowly depressed, injecting the cell suspension into the blood stream, taking care not to force the cells to shear in the syringe by excess pressure and not to move the needle out of the vein.

The animal was then monitored for signs of distress throughout the experiment. At the end of the experiment, animals were sacrificed and examined for the formation of tumours, usually in the lung tissue.

Figure 2.1: Detection of secondary P8 tumours in rat lung tissue stained with Indian ink



P8 transplantable tumour was grown on sub-cutaneously on the flank of AS rats. Rats with suspected lung metastases were anaesthetised using Halothane gas before draining blood from the heart by cardiac puncture. After the maximum amount of blood was drained from the heart, the rat was killed by dislocation of the neck. The heart and lungs were then removed and washed in PBS before being inflated with Indian ink solution, the trachea ligated and the tissue fixed in Feketes solution. The lung tissue stains black, leaving the secondary tumour tissue white as indicated by the arrow.

2.2.7.7 Intra-tumour injections

Animals were induced into anaesthesia using Halothane gas anaesthetic. The syringe was loaded with appropriate solution, taking care to expel air bubbles from the syringe as described above. Injections were made deep into the tumour, withdrawing the needle as the solution is expelled. No more than 0.2ml was administered during a single injection. If more solution needed administering, multiple injection sites were used, trying not to overlap injection tracts. Animals were then allowed to recover from anaesthesia.

2.2.7.8 Isolation of cells from tumour

The primary tumour load was harvested as described above when the tumour was between 0.5 – 0.75cm in diameter. The surrounding connective tissue was cut away and disguarded. The remaining tumour was then finely chopped using a scalpel in cell dispersion solution (collagenase type VII (Sigma) 500 units/ml in PBS pH7.4). Once the bulk of the tumour mass had been dispersed, the solution was incubated at 37°C for 1 hour. Cells were then washed in complete DMEM and seeded into a 75cm^2 tissue culture flask.

Cells were then passaged at least 5 times to remove the other non-tumour cells contaminating the culture.

2.2.8 Histochemistry techniques

2.2.8.1 Sectioning of tissue

2.2.8.1.1 Paraffin-wax embedding and sectioning of tissue

Tissue was excised and fixed in 4% neutral-buffered formalin (NBF, 4% formalin in PBS). Sections were then put into paraffin wax blocks using the tissue embedding center (Thermo-Shandon) and sections cut between 5-8μm in thickness, and put onto glass slides.

2.2.8.1.2 Cryostat sectioning of tissue

Snap frozen tissue was embedded in OCT compound (Raymond Lamb) at –60°C using the cryobar of a Shandon cryostat (Cryotome, Shandon

Scientific, UK). The embedded tissue was allowed to warm to between -14 and -10°C and sectioned at 10µm increments, Sections were placed on to BDH superfrost Gold microscope slides, air dried at room temperature for 10 min and stored at -70°C in air tight containers.

2.2.8.2 Histochemical staining of paraffin-wax sections

Histochemical staining was carried out only on paraffin wax sections. Therefore, all methods are preceded by de-waxing and re-hydrating of the tissue section.

2.2.8.2.1 De-waxing and re-hydration of paraffin-wax embedded tissue sections

Slides were carefully dipped into the following solutions:

1.	Xylene	2 min
2.	Xylene	1 min
3.	Absolute Industrial methylated spirits (IMS)	2 min
4.	Absolute IMS	1 min
5.	70% IMS in water	1 min
6.	Tap water	1 min

2.2.8.2.2 Gills Haematoxylin and Eosin No. 3

Sections were de-waxed and re-hydrated as described above. The slides are placed into:

1.	Gills Haematoxylin No.3 (after being filtered with Whatman fast filter paper No.4)	2 min
2.	Running tap water	2 min
3.	Borate (1% in dH_2O, disodium tetraborate)	Dip
4.	Tap water	Rinse

At this point, slides were observed under a microscope. If nuclei were too blue, they were dipped in acid alcohol (70% IMS, 1% conc HCl in dH_2O),

followed by a tap water rinse. If nuclei were not blue enough, the above steps were repeated.

5. Eosin (1% in dH_2O, after being filtered with Whatman fast filter paper No.4) 2 min
6. Tap water Rinse

The slides were then dehydrated and mounted as described below. After staining, the slides were viewed under a light microscope (Ziess, Axioskop). The nuclei appear blue and the cytoplasm stains pink.

2.2.8.2.3 Massons Trichrome stain for general collagens

Sections were de-waxed and re-hydrated as described above. The slides are placed into:

1. Gills Haematoxylin No.3 (after being filtered with Whatman fast filter paper No.4) 2 min
2. Running tap water 2 min
3. Borate (1% in dH_2O, disodium tetraborate) Dip
4. Tap water Rinse

At this point, slides were observed under a microscope. If nuclei were too blue, they were dipped in acid alcohol (70% IMS, 1% conc HCl in dH_2O), followed by a tap water rinse. If nuclei were not blue enough, the above steps were repeated.

5. Acid fuchsin (0.5% in 0.5% acetic acid in dH_2O) 5 min
6. dH_2O Rinse
7. Phosphomolybdic acid (1% in dH_2O) 5 min

At this point, the slides were thoroughly drained, but not left to dry out.

8. Methyl blue (2% in 2.5% acetic acid in dH_2O) 5 min

9.	dH_2O	Rinse
10.	Acetic acid (1% in dH_2O)	2 min

The slides were then dehydrated and mounted as described below. After staining, the slides were viewed under a light microscope (Ziess, Axioskop). Nuclei appear blue/black, cytoplasm/muscle/red blood cells appear red and collagen appears blue/green.

2.2.8.2.4 Dehydrating and mounting paraffin-wax embedded tissue sections

Slides were carefully dipped into the following solutions:

1.	70% IMS	Dip
2.	Absolute IMS	1 min
3.	Absolute IMS	2 min
4.	Xylene	1 min
5.	Xylene	2 min

The slides were then mounted in Xam wax-based mounting media.

2.2.8.3 Immunohistochemical staining of cryostat sections

To prevent false localisation this technique necessitates the removal of soluble tTGase, therefore it only localises insoluble (substrate bound) tTGase. All solutions prior to fixation were supplemented with the protease inhibitors 1mM leupeptin, 1mM benzamidine, 1mM pepstatin, 1mM phenylmethylsulfonyl fluoride (PMSF) and 10mM EDTA. Unfixed cryostat sections were brought to room temperature, washed twice with PBS (pH7.4) to remove soluble tTGase and then blocked for 1 hour at room temperature with 3% bovine serum albumen (BSA) in PBS (pH7.4). Sections were then washed twice with PBS (pH7.4) for 5 min and immunoprobed with 100µl of either 1:50 dilution of monoclonal anti-tTGase antibody (CUB7042, Neomarkers, Stratech Scientific, UK), 1:100 dilution of a monoclonal anti-fibronectin antibody (Sigma), or 1:2000 dilution of polyclonal anti-von

Willebrand factor (Sigma). Antibodies were diluted in 3% BSA containing the protease inhibitors and incubated at 4°C overnight. Sections were washed three times in PBS (pH7.4) and then fixed in -20°C methanol for 10 minutes. These sections were subsequently washed three times in PBS (pH7.4) before blocking again with 3% BSA for 1 hour at room temperature. Anti-mouse IgG antibody conjugated to FITC was diluted 1:1000 in 3% BSA and added to the sections for 2 hours at room temperature. Sections were then washed three times in PBS (pH7.4) and counter-stained with 5μg/ml propidium iodide (PI). Sections were washed again to remove excess PI and mounted with Vector-Shield fluorescent mounting media (Vector Laboratories, Peterborough, UK).

Sections were visualised using a Leica TCS NT confocal microscope (Leica). Settings were varied to obtain optimum picture quality.

2.2.9 Statistical analysis

The student's t-test for parametric distributions determined statistical significance of differences from controls.

Chapter 3

TISSUE TRANSGLUTAMINASE AND CELL DEATH IN THE MET B HAMSTER FIBROSARCOMA

3.1 Introduction

Fesus and colleagues first showed the involvement of tTGase in apoptosis or programmed cell death (Fesus *et al.*, 1987). This prompted others to investigate the role of tTGase in the apoptotic pathway further. One such group took a series of hamster fibrosarcoma cell lines of different metastatic potential and demonstrated that tTGase activity could be correlated to the number of detergent-insoluble ("apoptotic") bodies and ε-(γ-glutamyl) lysine crosslink found in the cells (Knight *et al.*, 1991). As a consequence of this and other groups work, the role of the enzyme was postulated to be in the formation of the SDS-insoluble "apoptotic body" found at the terminal stages of apoptosis. The bodies were thought to be important in preventing the release of cellular debris into the surrounding area, thus helping prevent an immune response. The role of tTGase in apoptosis has also been investigated by several researchers worldwide, who argue a more regulatory role for tTGase in the apoptotic pathway (Melino and Piacentini, 1998). Melino and others found that increasing the amount of tTGase in human neuroblastoma cells made the cells more susceptible to death by apoptosis (Melino *et al.*, 1994) when treated with apoptosis inducing agents.

Owing to the potential importance of tTGase in cell death, a number of studies have been focussed on the factors that regulate its expression. Previous investigations have shown that retinoids (Davies *et al.*, 1985), TGF-β_1 (Jetten and Shirley, 1986) and interleukin 6 (Suto *et al.*, 1993) are all capable of inducing tTGase expression in various cell lines. The glucocorticoid dexamethasone is another agent that can be used to induce tTGase expression, as recent isolation and characterisation of the human tTGase gene promoter had revealed a glucocorticoid-response element (Lu *et al.*, 1995).

In the past few years, the understanding of the apoptotic pathway has increased considerably and new methods of apoptosis detection are currently available. It was decided to investigate the formation of these detergent

insoluble apoptotic bodies and their validity as a marker of apoptosis by comparing them to the newer methods of apoptosis detection. In addition, investigations were also carried out to assess if tTGase induction, leading to cell death, could be used as a therapeutic agent against tumours.

The model chosen was the Met B hamster fibrosarcoma. This tumour cell line, when transfected with tTGase cDNA had previously been shown to fail to form primary tumours when injected subcutaneously into hamsters (Johnson *et al.*, 1994). How this loss of malignancy comes about is still unknown, although this phenomenon is also seen in other tumour models studied in this Thesis.

3.2 Results

3.2.1 Induction of tTGase activity by dexamethasone in the Met B cell line

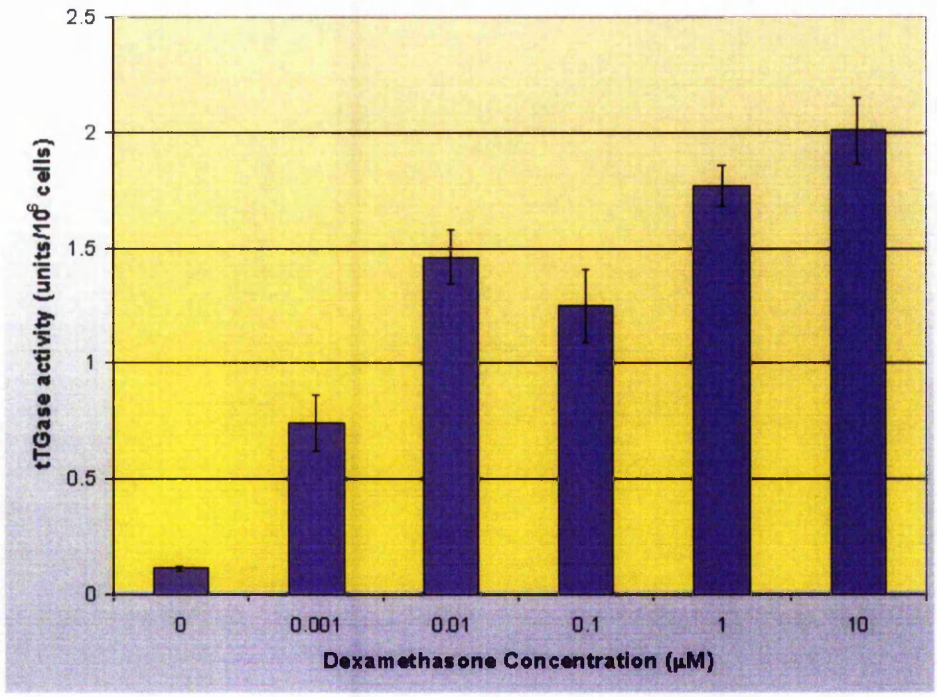
Following dose-response experiments involving treatment of the Met B fibrosarcoma cell line with dexamethasone in a concentration range of 0-10μM (Figure 3.1), it was found that near optimal induction of tTGase could be obtained by treatment with 1μM dexamethasone for 48 hours.

3.2.2 Mode of tTGase induction by dexamethasone in the Met B cell line

To determine whether this increase in tTGase activity in the Met B fibrosarcoma cell line was due to an upregulation of gene transcription or a post-translational modification of the tTGase protein, cells were treated with the transcription inhibitor actinomycin D and the protein translation inhibitor cyclohexamide in the presence and absence of dexamethasone. The levels of tTGase antigen was then determined by fractionating cell homogenates by SDS-PAGE, Western blots and then immunoprobing with CUB7402 α-tTGase mAb. Figure 3.2 shows an increase in tTGase antigen with dexamethasone treatment. This is blocked by both 1μg/ml actinomycin D and 10μg/ml cyclohexamide.

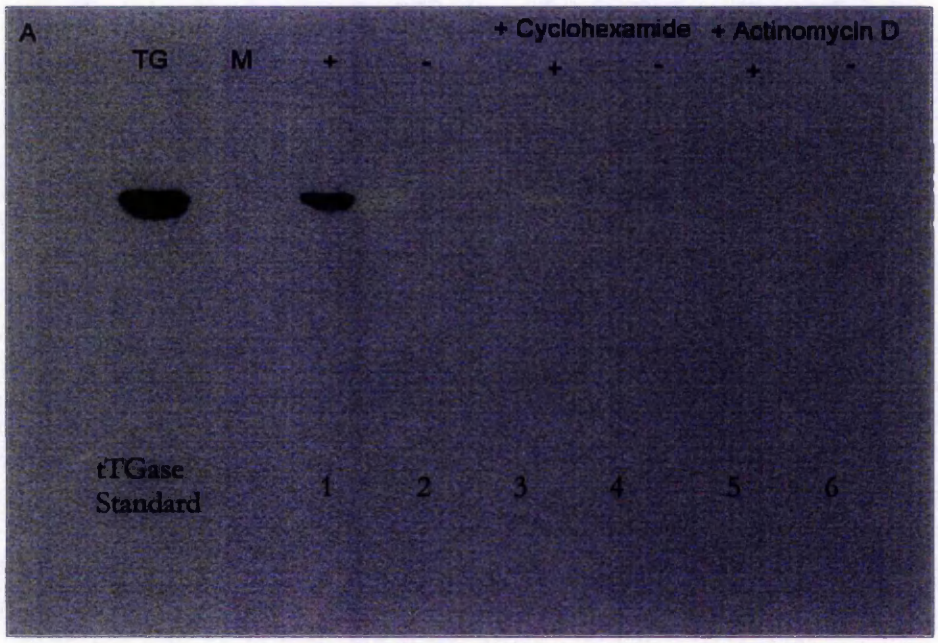
To confirm that the induction was due to *de novo* synthesis, tTGase mRNA levels were also determined by Northern blot analysis. The autoradiograph shown in figure 3.3 shows an increase in tTGase mRNA in Met B after treatment with dexamethasone.

Figure 3.1: Dose-response of Met B cell line to dexamethasone induction of tTGase.



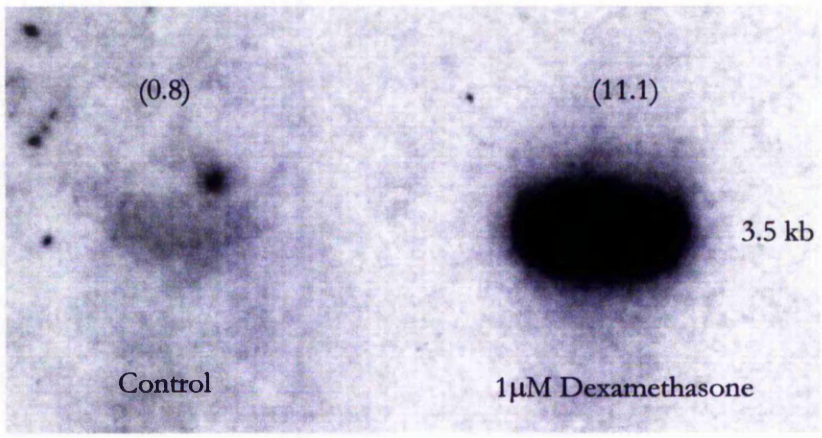
Confluent (60-70%) cell cultures of Met B were treated for 48 hours with various doses of dexamethasone and then harvested using trypsin and counted on a haemocytometer. Aliquots ($1x10^6$ cells) were homogenised and the tTGase activity was determined by the incorporation of [^{14}C]putrescine into N,N'-dimethylcasein. Data are mean ± S.E.M from three experiments.

Figure 3.2: Effects of inhibitors on dexamethasone induction of tTGase in Met B cells



Key: Lanes 1,3,5 + dexamethasone; 2,4,6 – dexamethasone. Lanes 3+4 + cyclohexamide; 5+6 + actinomycin D. Cells were grown in the presence or absence of 1μM dexamethasone for 48 hours and in the presence or absence of 10μg/ml cyclohexamide or 1μg/ml actinomycin D. Cells were then harvested by trypsinization and the cell number determined. $1x10^6$ cells were homogenised and equivalent protein loaded onto a 10% (w/v) polyacrylamide gel, electrophoresed and electroblotted onto Hybond C Super Nylon. The Western blot was immunoprobed with CUB7402 α-tTGase mAb as described in the Materials & Methods chapter. Guinea-pig liver tTGase was also run as a standard.

Figure 3.3: Northern blot analysis of mRNA from dexamethasone treated Met B cells



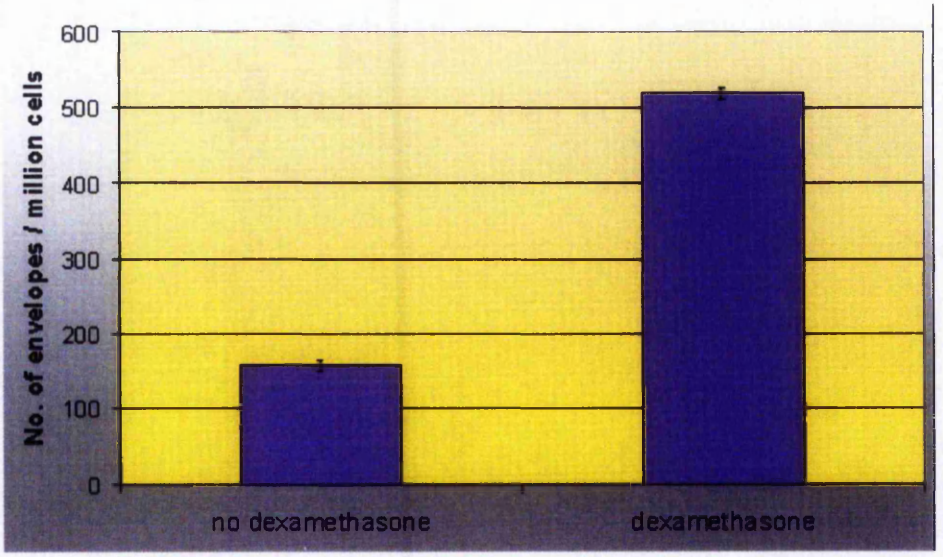
Total RNA was extracted from a 90% confluent 75cm^2 flask of Met B previously treated with 1μM dexamethasone or grown in normal media for 48 hours. The Northern blot was probed using a mouse [^{32}P]dCTP random-primed probe specific for tTGase as described in the Materials & Methods chapter. Volume densities corrected for RNA loading are shown in parentheses.

3.2.3 Dexamethasone-induced cell death in the Met B fibrosarcoma cell line

Met B cells treated with dexamethasone have been shown to give rise to an increase in apoptosis as determined by the formation of SDS-insoluble apoptotic bodies (Johnson, 1995). Following a similar protocol, it can be shown that the percentage increase in SDS-insoluble apoptotic body formation following treatment with 1μM dexamethasone was found to be 329%. These results are shown in figure 3.4.

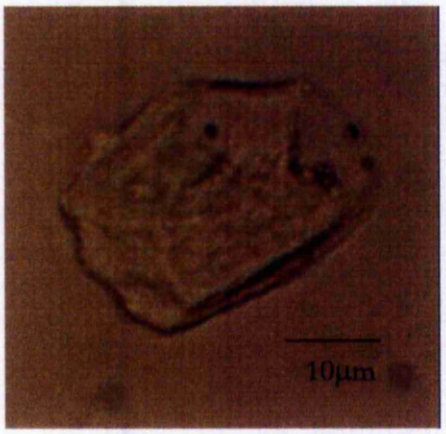
The increase in SDS-insoluble "apoptotic body" formation has however now become a questionable method of apoptosis detection, as new methods have become available. One of the purposes of the following chapter was to determine how this method compared with more recent technology in order to asses its validity. It has now become common practice to determine whether a cell is dying via a classical apoptotic pathway using a combination of 2 or 3 different methods of apoptosis detection. Levels of apoptosis in Met B cells treated with dexamethasone were therefore evaluated using 3 different criteria: DNA fragmentation; activation of caspase-3 (CPP32), an ICE protease activated in early apoptosis; and ADP:ATP ratios, which give an indication of both the viability of the cells within the sample as well as the method by which cells are dying (i.e. necrosis or apoptosis) if required.

Figure 3.4a: Number of SDS-insoluble shells produced by Met B cells when treated with 1 μM dexamethasone for 48 hours



SDS-insoluble shells isolated from cells grown in the presence or absence of 1 μM dexamethasone for 48 hours. Adherent cells were recovered from the plate using trypsin and pooled with those present in the supernatant. Total cells number was determined using a haemocytometer and detergent insoluble bodies isolated by boiling the cells in SDS as described in the Materials & Methods chapter. The data represent means ± S.E.M. from three experiments. The experiment showed a significant difference in the number of shells isolated ($p \leq 0.05$).

Figure 3.4b: Picture of an SDS-insoluble envelope



Picture of SDS-insoluble envelope isolated from cells grown in the presence of 1μM dexamethasone for 48 hours. Detergent insoluble bodies were isolated by boiling the cells in SDS as described in the Materials & Methods Chapter.

3.2.4 Alternative Apoptosis Detection Methods

Met B cells were grown on glass slides and were treated with 1μM dexamethasone for 48 hours prior to ApoTag staining (a method which detects DNA fragmentation), in order to allow time for the DNA to fragment. The staining was carried out as detailed in the Materials & Methods chapter. Cells isolated from the supernatant were also collected by centrifugation and air-dried onto glass slides and stained in the same way. Figure 3.5 shows photographs of the ApoTag staining. The staining showed no increase in apoptosis with dexamethasone treatment.

In order to assess earlier stages of apoptosis, two other methods of apoptosis detection were employed: Caspase-3 induction and ADP:ATP ratios. The ApoAlert kit (Clontech) measures the activation of caspase-3 by providing a colourimetric substrate, which is cleaved and can be used to give an absorbance reading on a spectrophotometer. The results of this experiment (figure 3.6) showed no increase in apoptosis following treatment with 1μM dexamethasone. In fact, a significant decrease in caspase-3 activity was observed following dexamethasone treatment. The ATP readings from the Apoglow kit (LumiTech) showed no reduction in viability in the cells treated with dexamethasone (Figure 3.7) and the ADP:ATP ratios show the cells are still proliferating.

These methods of apoptosis detection show no increase in apoptosis in the cells treated with dexamethasone, when compared with the non-dexamethasone treated cells.

To further prove that the SDS-insoluble bodies detected were not a product of apoptotic cell death, Met B cells stably transfected with the anti-apoptotic gene bcl-2 in the vector pSVbcl2 and control cells transfected with just pSVneo were analysed. Dexamethasone induction of tTGase was of similar levels to the wild type (Figure 3.8). Ionomycin was added to the samples to

induce apoptosis. No apoptosis was observed in the bcl-2 clones as measured by caspase-3 induction, however, in the control clone, caspase-3 induction was of a similar level to that of the wild type Met B (Figure 3.9).

The clones were then treated with 1μM dexamethasone for 48 hours before harvesting and isolating the SDS-insoluble material as described in the Methods section. In all cases, the number of SDS-insoluble bodies recovered was comparable with the wild type Met B cells (Figure 3.10)

Figure 3.5: ApoTag staining of Met B cells treated with 1μM dexamethasone for 48 hours

Cells were grown on glass slides in the presence or absence of 1μM dexamethasone for 48 hours. Cells in the supernatant were collected and air-dried onto separate slides. Adherent and floating cells were assayed for DNA fragmentation using the ApoTag (Oncor) kit in accordance with manufacturers instructions. Slides were viewed using a Leica confocal microscope. Settings were varied to get optimum picture quality. (A) Adherent control cells; (B) Floating control cells; (C) Adherent dexamethasone treated cells; (D) Floating dexamethasone treated cells.

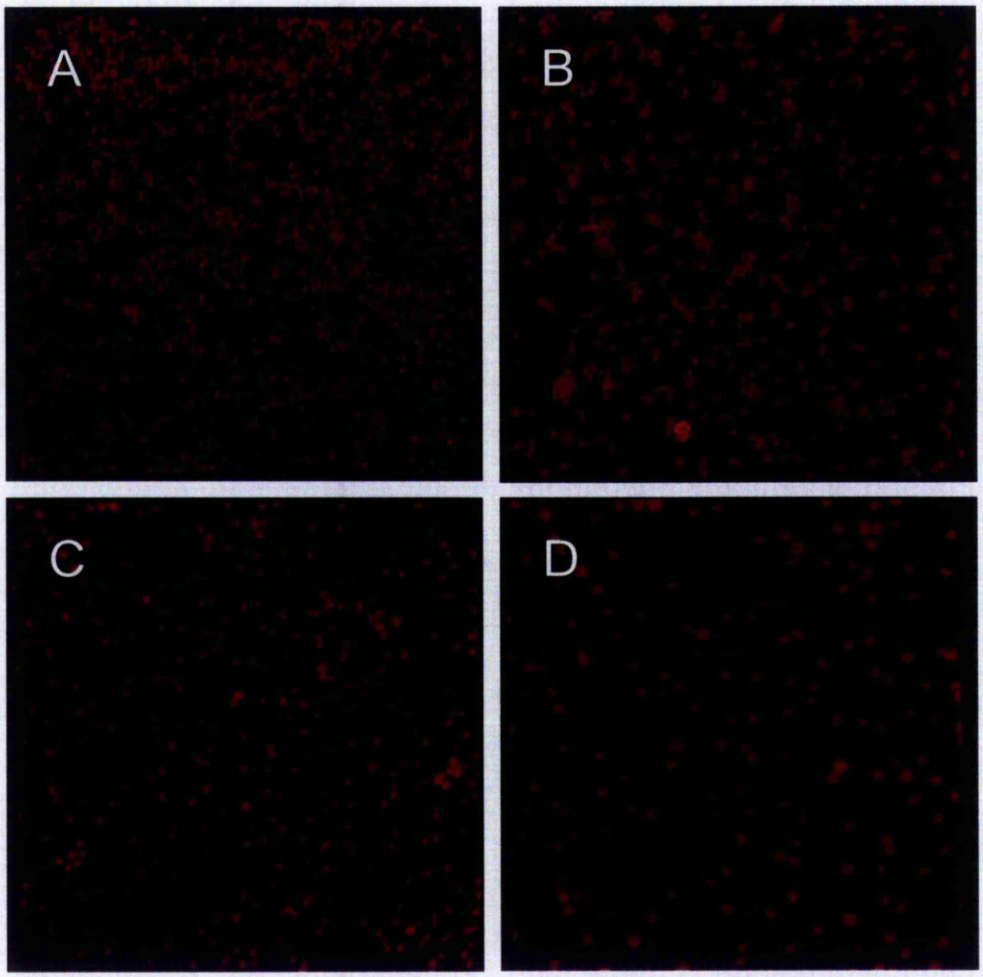
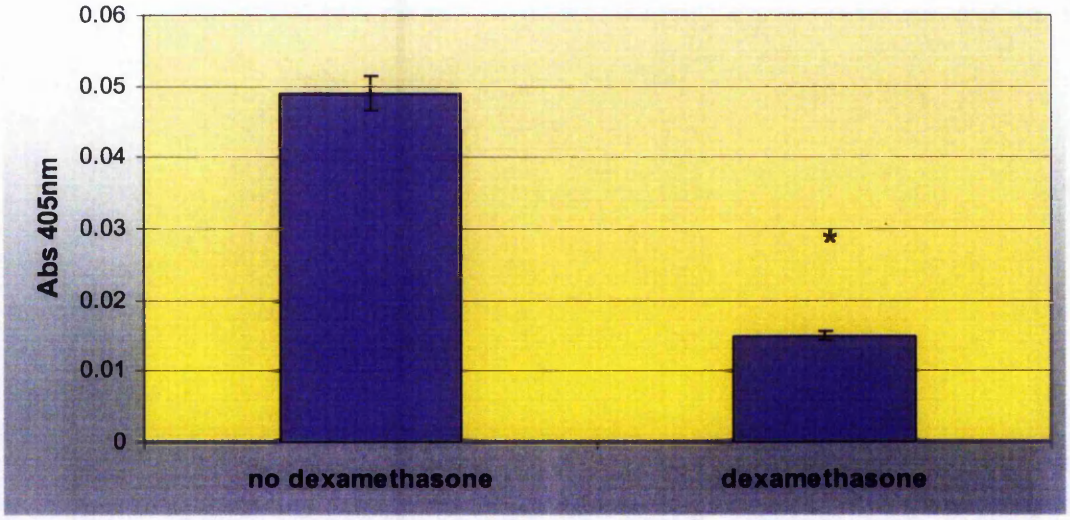

A
B
C
D

Figure 3.6: Measurement of Caspase-3 (CPP-32) activity in Met B cells treated with 1 μM dexamethasone for 48 hours

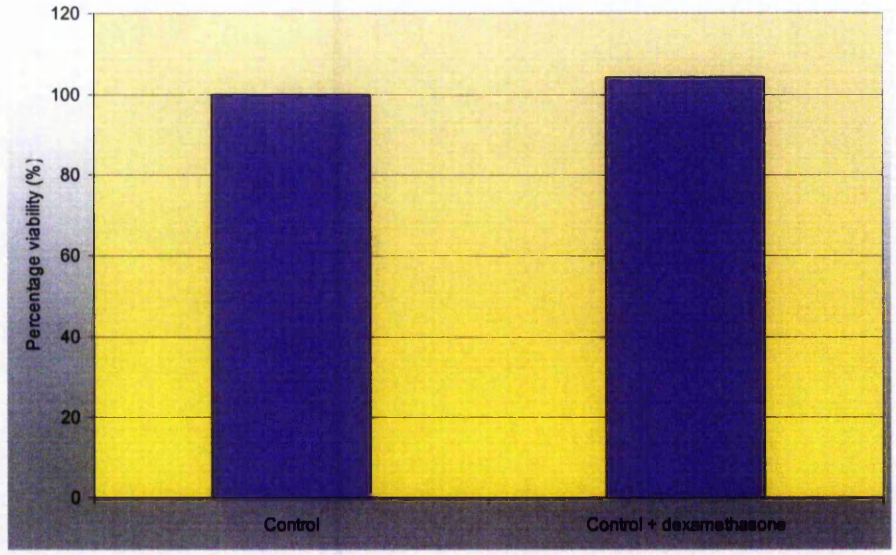


*Caspase-3 activity expressed as absorbance measured at 450nm, measured using the ApoAlert kit from Clontech. Cells were grown in the presence or absence of 1μM dexamethasone for 48 hours. Cells were collected and counted. $2x10^6$ cells were spun down and assayed in accordance with manufacturers instructions. The data represent means ± S.E.M. from three experiments. * shows $p \leq 0.05$.*

Figure 3.7: (a) Percentage Viabilities of Met B cells with and without 1μM dexamethasone as measured by total ATP levels & (b) ADP:ATP ratios of Met B cells with and without 1μM dexamethasone

Cells were grown in 96-well plates for 48 hours in the presence or absence of 1μM dexamethasone and apoptotic agents for 36 hours. Cells were assayed using the ApoGlow (Lumitech) kit which determines the state of the cell by comparing the levels of ATP and ADP. Protocol was carried out according to manufacturers instructions. (a) shows the percentage viability of cells calculated from the ATP measurements. (b) shows the ADP:ATP ratios obtained from the assay. The data represent means ± S.E.M. from three experiments. The results were not significantly different ($p \leq 0.05$).

(a)


120
100
80
60
40
20
0
Percentage viability (%)
Control
Control + dexamethasone

(b)

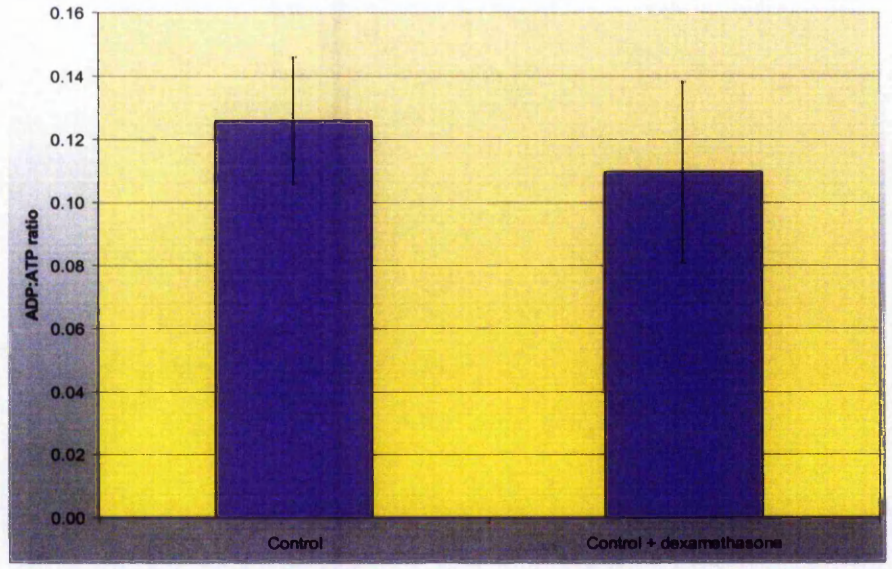
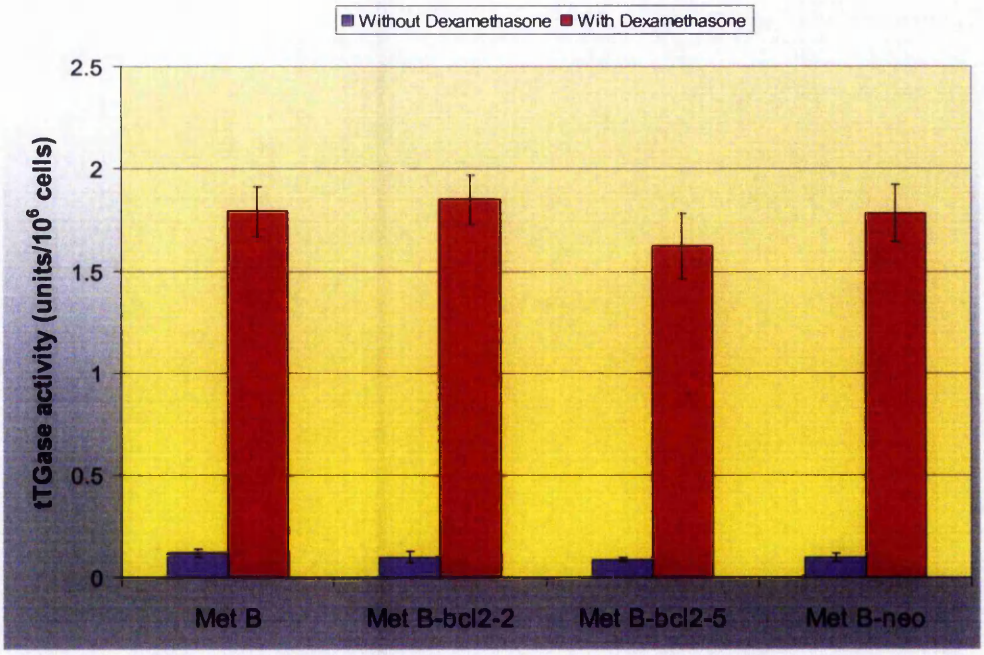
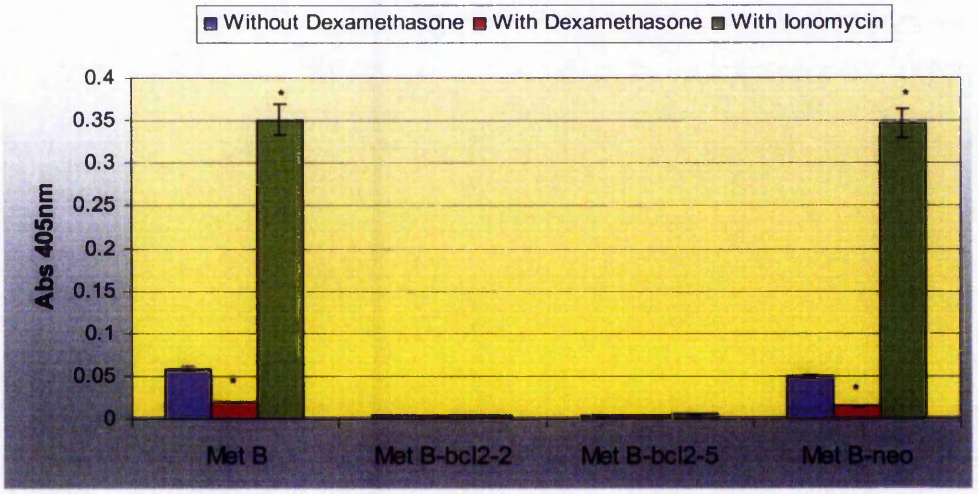

0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
ADP:ATP ratio
Control
Control + dexamethasone

Figure 3.8: Dexamethasone induction in Met B clones



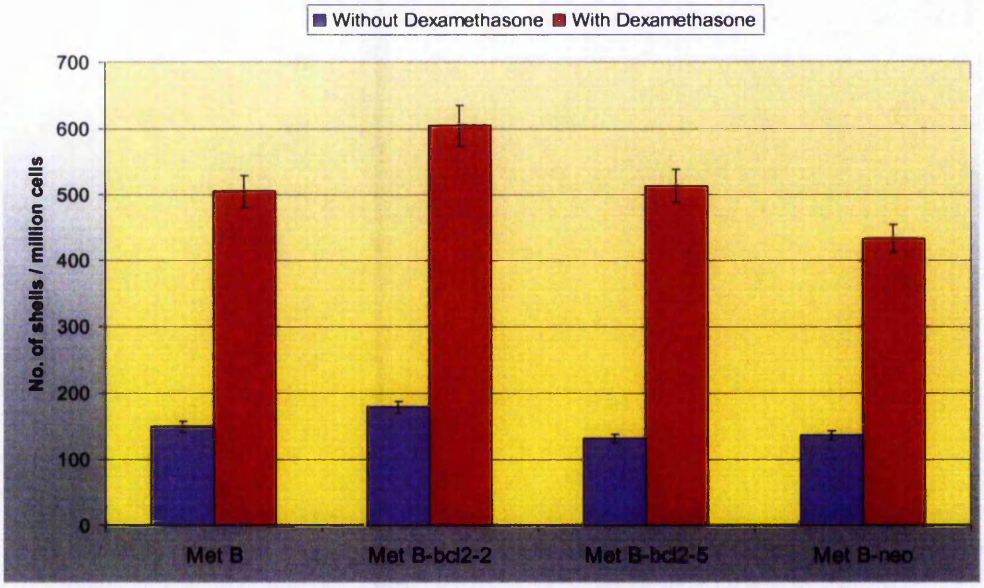
Cells were grown with or without 1μM dexamethasone for 48 hours and then harvested using trypsin and counted on a haemocytometer. Aliquots ($1x10^6$ cells) were homogenised and the tTGase activity was determined by the incorporation of [^{14}C]putrescine into N,N'-dimethylcasein. Data are mean ± S.E.M from three experiments. All cell lines showed a significant increase in tTGase activity ($p \leq 0.05$).

Figure 3.9: Ionomycin-induced apoptosis in Met B clones



*Caspase-3 activity of Met B clones measured using the ApoAlert kit from Clontech. Cells were grown in the presence or absence of 1μM dexamethasone for 48 hours and/or 20μM ionomycin. Cells were collected and counted. $2x10^6$ cells were spun down and assayed in accordance with manufacturers instructions. The data represent means ± S.E.M. from three experiments. * shows statistically significant difference from the control ($p \leq 0.05$).*

Figure 3.10: Number of SDS-insoluble shells formed by Met B clones when treated with 1μM dexamethasone for 48 hours



SDS-insoluble shells isolated from cells grown in the presence or absence of 1μM dexamethasone for 48 hours. Adherent cells were recovered from the plate using trypsin and pooled with those present in the supernatant. Total cells number was determined using a haemocytometer and detergent insoluble bodies isolated by boiling the cells in SDS as described in the Materials & Methods chapter. The data represent means ± S.E.M. from three experiments. The experiment showed a significant difference in the number of shells isolated following dexamethasone treatment in each cell line ($p \leq 0.05$).

3.2.5 Cellular damage by trypsinisation

As the cells were not dying by classical apoptosis with dexamethasone treatment, yet there was an increase in the formation of SDS-insoluble "shells", it was decided to investigate the mechanism of their appearance. It was concluded that tTGase must be activated in the dying cells at some point during the experiment due to loss of Ca^{2+} homeostasis. The most likely cause of this activation was with limited membrane damage. It was deduced that this was most likely to happen during the cell trypsinisation process, prior to the solubilisation of the cells in detergent. To investigate the effect of trypsinisation on the cells, cells were incubated with a fluorescently labelled tTGase primary amine substrate (fluorescein-cadaverine) immediately after harvesting. Any free labelled cadaverine not covalently incorporated would be washed away during the methanol treatment of the process. Only labelled cadaverine incorporated into glutamine residues by tTGase would remain and would be visible under a fluorescence microscope. The cells treated with 1μM dexamethasone for 48 hours showed a three-fold increase in incorporation of fluorescein-cadaverine compared to the non-induced controls (Figure 3.11 A & B, and Figure 3.12; 22.5% ± 3.0% in dexamethasone treated cells, compared to 7.2% ± 0.8% in uninduced Met B cells; data expressed as mean ± S.E.M. from four experiments).

The fluorescent cells were boiled in SDS as described in the Methods chapter. The resulting fluorescent bodies were also visible under the confocal microscope (Figure 3.11 C), indicating a high level of tTGase associated cross-linking. When counted, the increase in fluorescent shells was found to be three-fold (figure 3.12), which correlates to the three-fold increase in "apoptotic body" formation observed in the initial experiment.

Figure 3.11: Comparison of cellular damage by trypsinization in Met B cells treated with 1μM dexamethasone and untreated cells as indicated by the covalent incorporation of fluorescein-cadaverine

Cells were incubated in the absence (A) or presence (B) of 1μM dexamethasone for 48 hours. Cells were trypsinized using standard protocol, pelleted and incubated in the presence of 0.5mM fluorescein-cadaverine for 45 mins at 37°C. An aliquot of cells was air-dried onto a glass slide, fixed in methanol (-20°C), washed and viewed using a Leica confocal microscope. SDS-insoluble insoluble bodies (C) were isolated from the remaining cells from the aliquot seen in (B) and isolated as in the Materials & Methods chapter, and viewed under the confocal microscope.

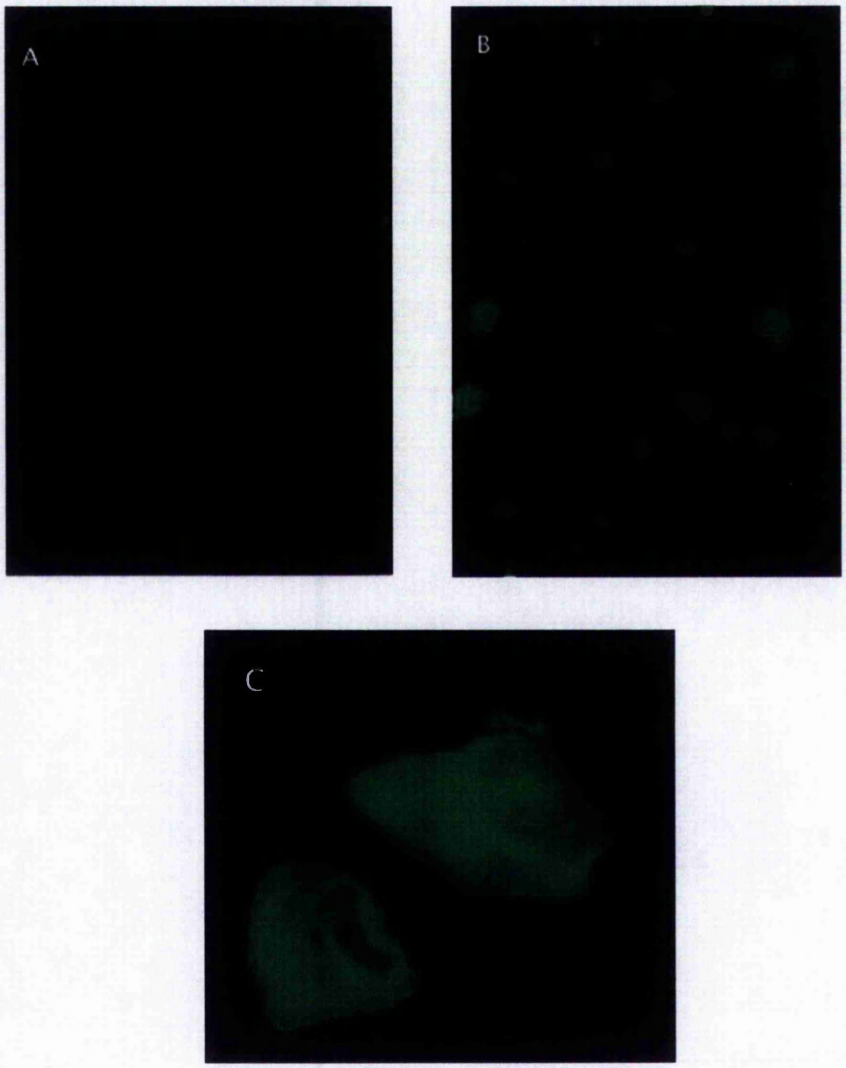
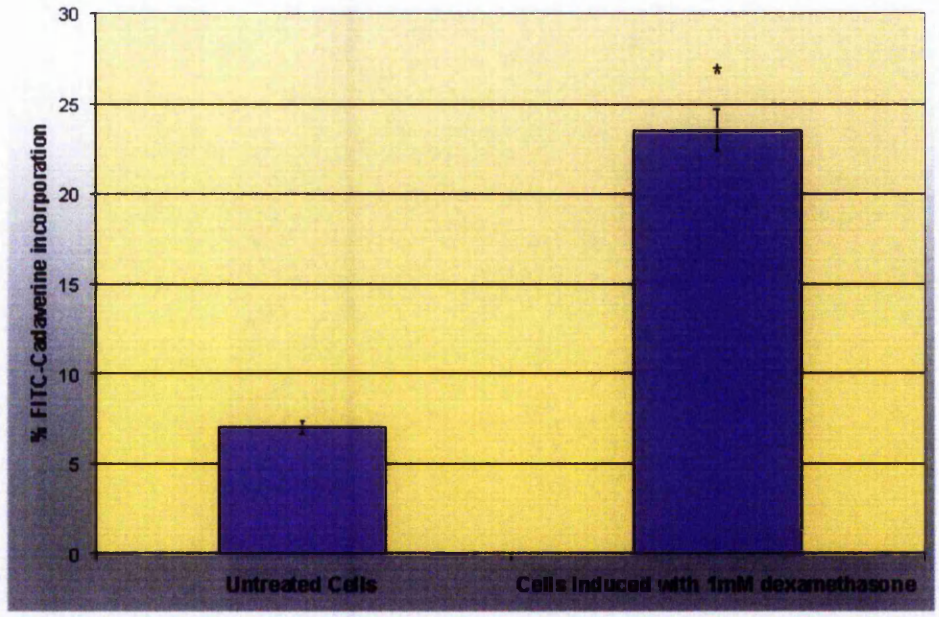

A
B
C

Figure 3.12: Extent of fluorescein-cadaverine incorporation in dexamethasone-induced and non-induced Met B cells



*Cells were treated for 48 h with 1μM dexamethasone. Adherent cells were recovered from the plate using trypsin and pooled with those present in the supernatant. FITC-Cadaverine was added for 45mins. Total cell number was determined using a haemocytometer and detergent-insoluble bodies isolated by boiling the cells in the presence of reducing SDS as described in the Materials and methods section. Detergent-insoluble bodies were determined from scanned photographs of 3 fields of view and analysed using the Aequitas Image analysis software. Data represent means S.E.M. from three experiments. * shows significant difference in result ($p \leq 0.05$).*

3.2.6 Transglutaminase-associated cell death

The data so far indicates that following limited cell damage, tTGase can be activated, resulting in the death of cells and the parallel formation of highly crosslinked envelopes comparable in size to the original cells. The data also indicates that this form of cell death does not involve the activation of caspase-3 or lead to DNA fragmentation. In order to further characterise the new form of cell death, Met B cells were treated with various apoptosis inducing agents in the presence and absence of 1μM dexamethasone. Cells were assayed for apoptosis by DNA fragmentation, caspase induction and ADP:ATP ratio. Cells were treated with different agents that induce apoptosis at different stages of the cell-cycle. Actinomycin D causes apoptosis during the G_2/M stage, camptothecin during the S-phase, ionomycin during the G_0/G_1 phase. Cells without dexamethasone induction of tTGase showed an increase in apoptosis when treated with all apoptotic agents (Figures 3.13 - 3.17). Cells treated with actinomycin D and camptothecin in the presence of dexamethasone also showed an increase in apoptosis, measured by caspase activity, ADP:ATP ratio and DNA fragmentation. However, cells treated with dexamethasone and ionomycin did not exhibit the same levels of apoptosis (Figures 3.13 - 3.17), but in fact showed a reduced level of apoptosis.

Figure 3.13: Apoptag staining of Met B cells treated with 3nM Actinomycin D in the presence and absence of 1μm dexamethasone

Cells were incubated in the presence of 3nM actinomycin D for 36 hours (apoptotic agent added 12 hours after the dexamethasone induction). Cells were grown on glass slides in the presence or absence of 1μM dexamethasone for 48 hours. Cells in the supernatant were collected and air-dried onto separate slides. Adherent and floating cells were assayed for DNA fragmentation using the ApoTag (Oncor) kit in accordance with manufacturers instructions. Slides were viewed using a Leica confocal microscope. Settings were varied to get optimum picture quality. (A) Adherent cells; (B) Floating cells; (C) Adherent dexamethasone treated cells; (D) Floating dexamethasone treated cells. Arrows shows examples of positive DNA fragmentation staining.

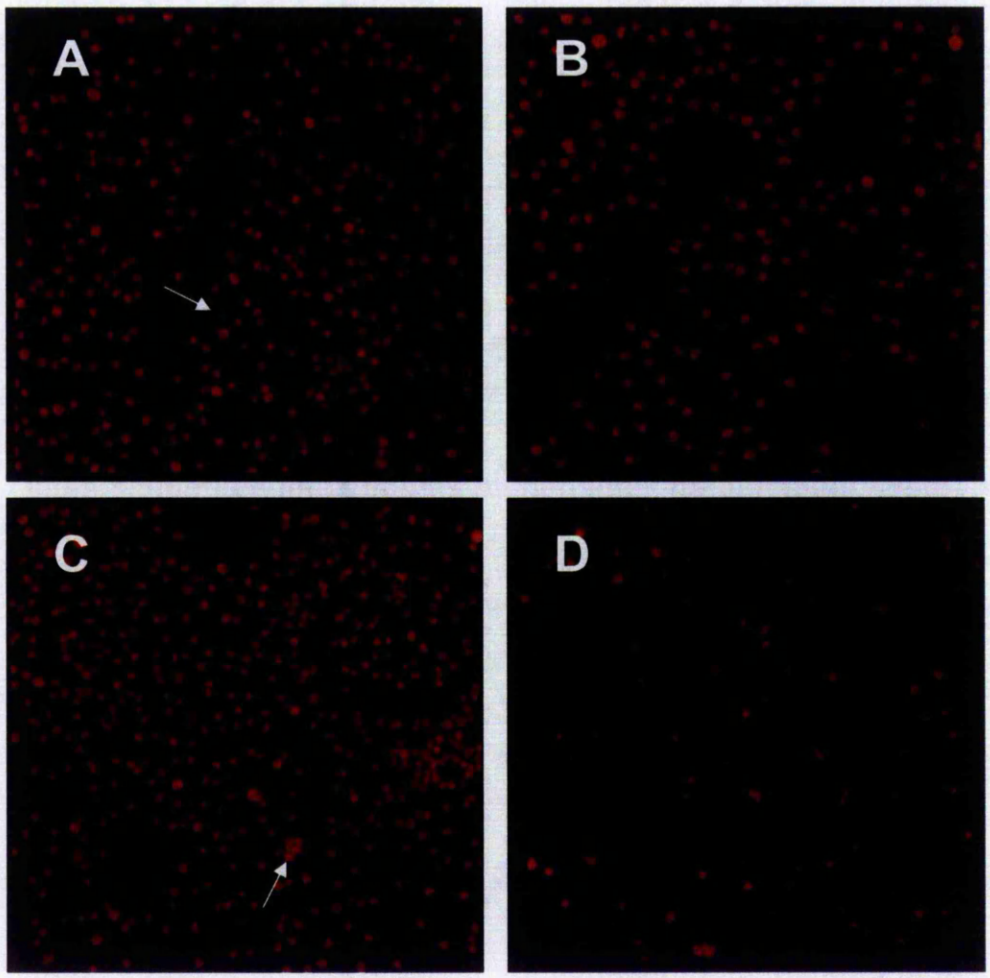

A
B
C
D

Figure 3.14: Apoptag staining of Met B cells treated with 0.7μM camptothecin in the presence and absence of 1μm dexamethasone

Cells were incubated in the presence of 0.7μM camptothecin for 36 hours (apoptotic agent added 12 hours after the dexamethasone induction). Cells were grown on glass slides in the presence or absence of 1μM dexamethasone for 48 hours. Cells in the supernatant were collected and air-dried onto separate slides. Adherent and floating cells were assayed for DNA fragmentation using the ApoTag (Oncor) kit in accordance with manufacturers instructions. Slides were viewed using a Leica confocal microscope. Settings were varied to get optimum picture quality. (A) Adherent cells; (B) Floating cells; (C) Adherent dexamethasone treated cells; (D) Floating dexamethasone treated cells. Arrows show apoptotic cells.

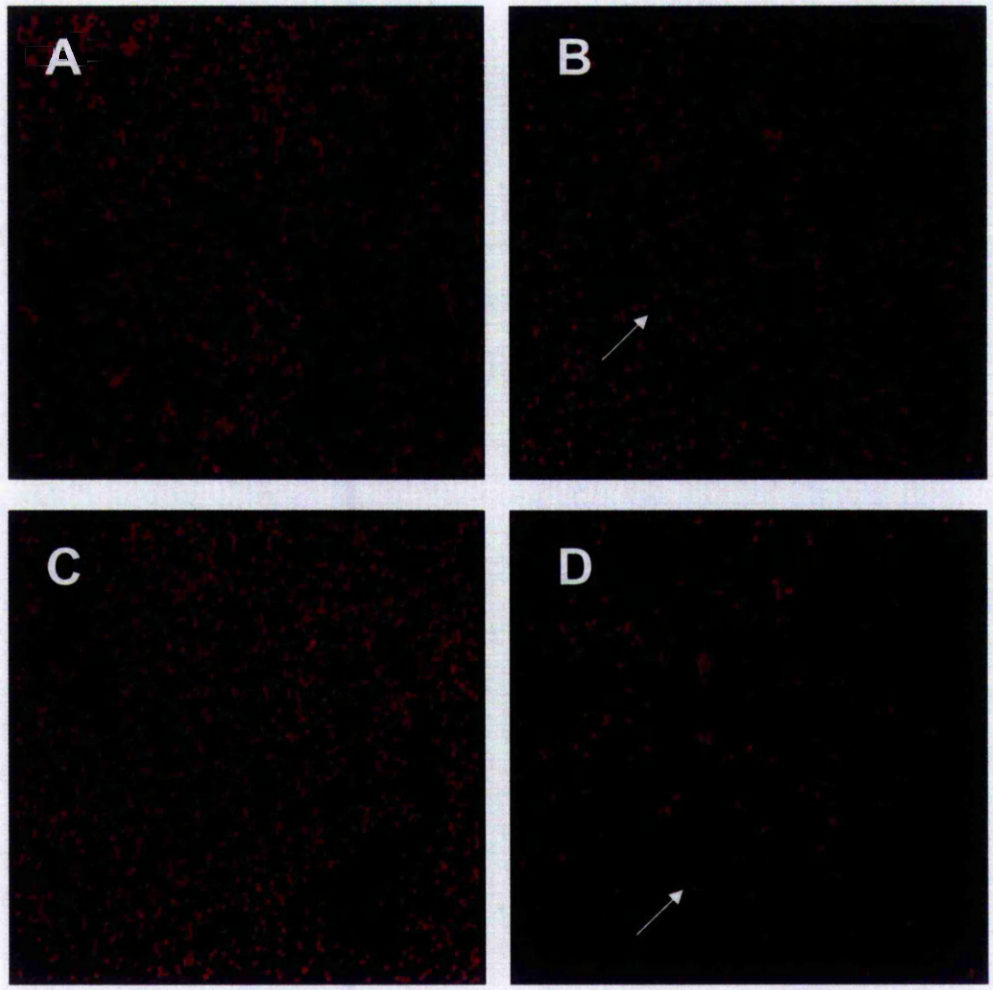

A
B
C
D

Figure 3.15: Apoptag staining of Met B cells treated with 20μM ionomycin in the presence and absence of 1μm dexamethasone

Cells were incubated in the presence of 20μM ionomycin for 36 hours (apoptotic agent added 12 hours after the dexamethasone induction). Cells were grown on glass slides in the presence or absence of 1μM dexamethasone for 48 hours. Cells in the supernatant were collected and air-dried onto separate slides. Adherent and floating cells were assayed for DNA fragmentation using the ApoTag (Oncor) kit in accordance with manufacturers instructions. Slides were viewed using a Leica confocal microscope. Settings were varied to get optimum picture quality. (A) Adherent cells; (B) Floating cells; (C) Adherent dexamethasone treated cells; (D) Floating dexamethasone treated cells. Arrows show examples of apoptotic cells.

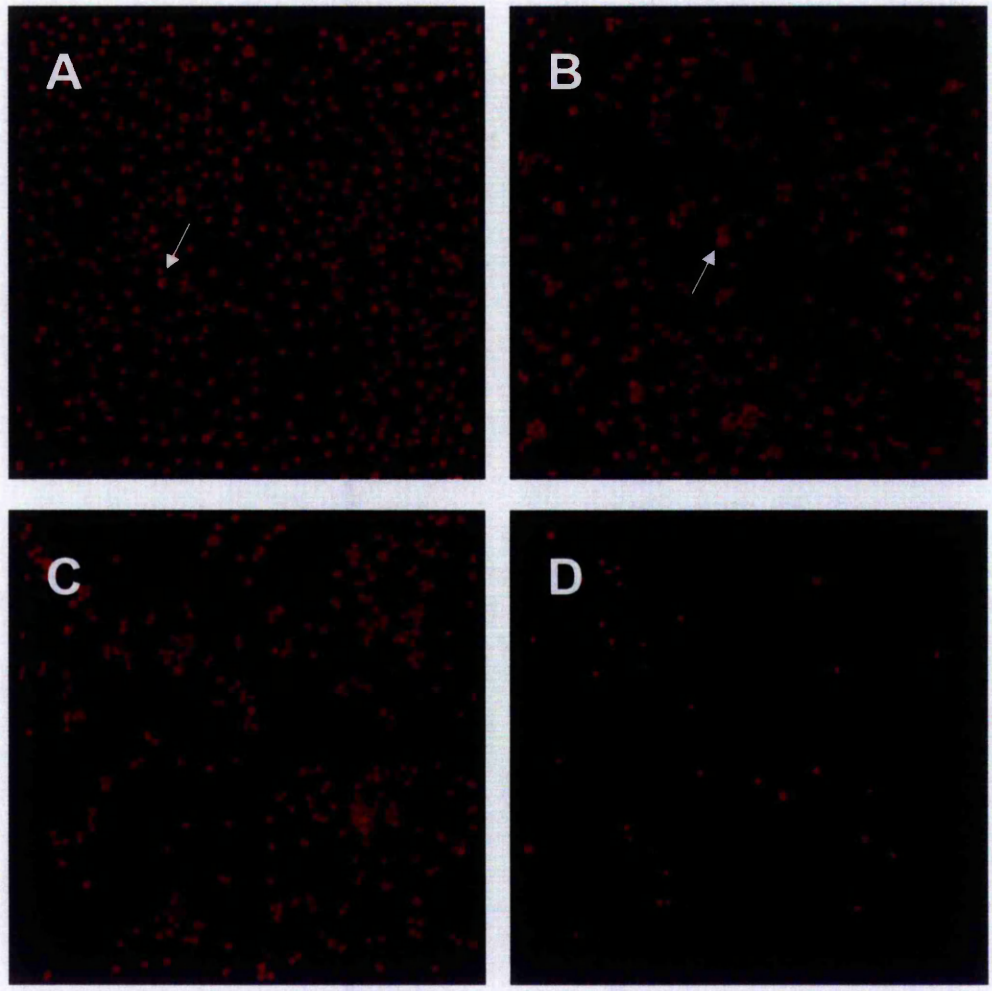
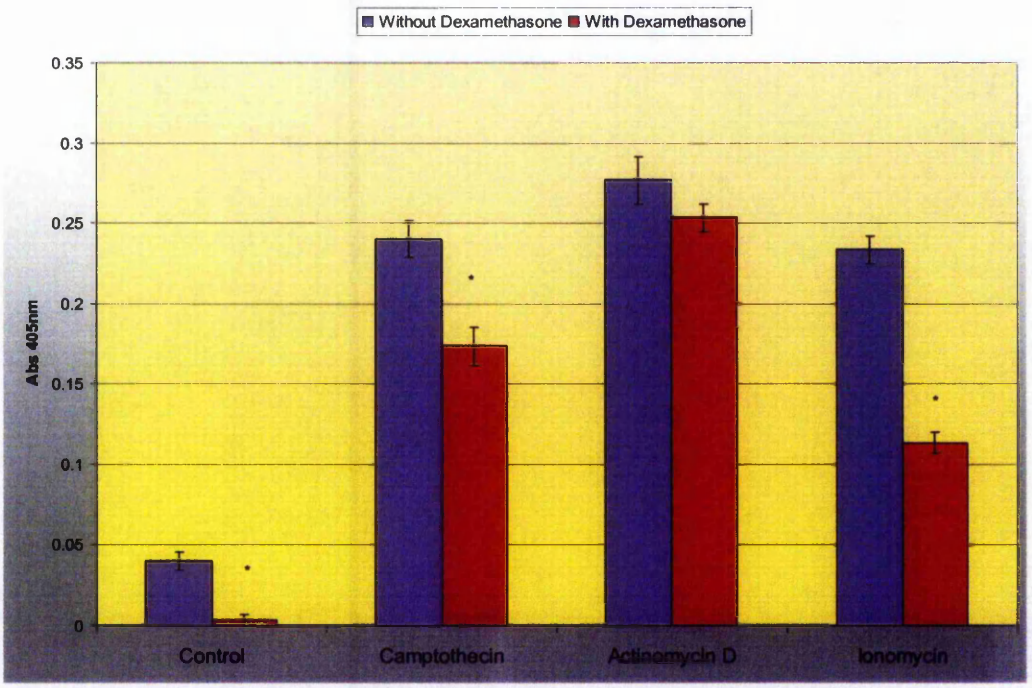

A
B
C
D

Figure 3.16: Measurement of Caspase-3 activity in Met B cells treated with 1 μM dexamethasone for 48 hours together with various apoptotic agents for 36 hours

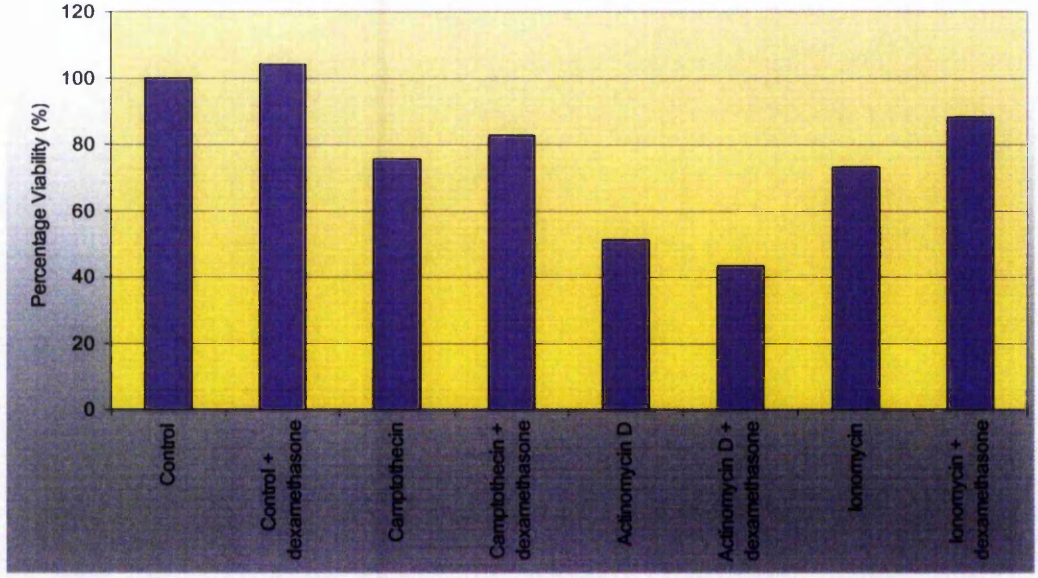


*Caspase-3 activity measured using the ApoAlert kit from Clontech. Cells were grown in the presence or absence of 1μM dexamethasone for 48 hours and apoptotic agents for 36 hours (apoptotic agent added 12 hours after the dexamethasone induction). Cells were collected and counted. $2x10^6$ cells were spun down and assayed in accordance with manufacturers instructions. The data represent means ± S.E.M. from three experiments. *'s show significant differences between samples treated with dexamethasone compared to untreated cells ($p \leq 0.05$).*

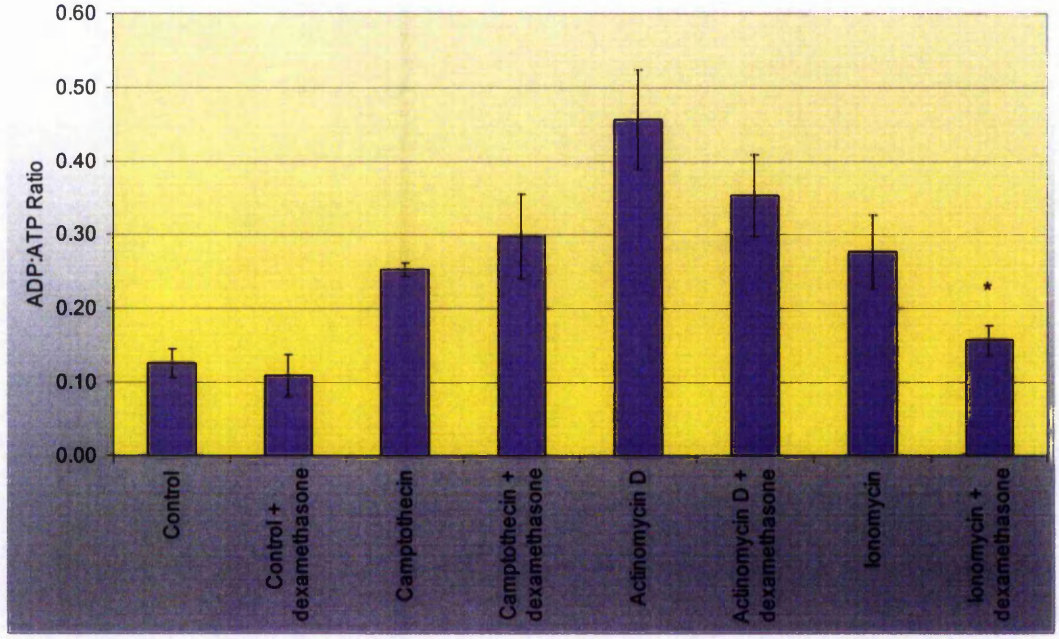
Figure 3.17: (a) Percentage Viabilities measured by total ATP levels of Met B cells treated with 1μM dexamethasone and various apoptotic agents & (b) ADP:ATP ratios of Met B cells treated with 1μM dexamethasone and various apoptotic agents

*Cells were grown in 96-well plates for 48 hours in the presence or absence of 1μM dexamethasone and apoptotic agents for 36 hours. Cells were assayed using the ApoGlow (Lumitech) kit which determines the state of the cell by comparing the levels of ATP and ADP. Protocol was carried out according to manufacturers instructions. (a) shows the percentage viability of cells calculated from the ATP measurements. (b) shows the ADP:ATP ratios obtained from the assay. The data represent means ± S.E.M. from three experiments. * shows significant difference between dexamethasone treated and untreated samples ($p \leq 0.05$).*

(a)


Percentage Viability (%)
120
100
80
60
40
20
0
Control
Control + dexamethasone
Camptothecin
Camptothecin + dexamethasone
Actinomycin D
Actinomycin D + dexamethasone
Ionomycin
Ionomycin + dexamethasone

(b)


ADP:ATP Ratio
0.60
0.50
0.40
0.30
0.20
0.10
0.00
*
Control
Control + dexamethasone
Camptothecin
Camptothecin + dexamethasone
Actinomycin D
Actinomycin D + dexamethasone
Ionomycin
Ionomycin + dexamethasone

3.2.7 Studies using solid tumours

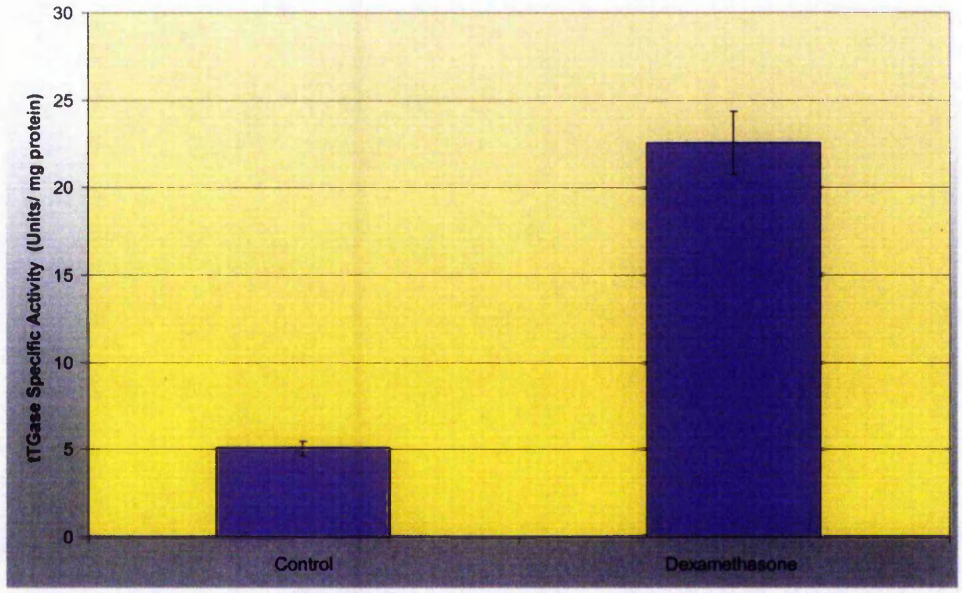
3.2.7.1 Induction of transglutaminase within the Met B fibrosarcoma

The data so far indicates that elevated tTGase levels and limited plasma membrane damage induces a new form of cell death which appears different to both apoptosis and necrosis. The new form of transglutaminase-associated cell death appears to occur more quickly than apoptosis.

Having shown that a new form of cell death could be induced *in vitro*, the induction of transglutaminase-associated cell death *in vivo* should be investigated. Met B tumours were grown on the flank of Golden Syrian hamsters.

As the half-life of dexamethasone in the available hamsters was calculated to be 3.5 hours (Buckley, *personal communication*), systemic intraperitoneal (IP) injections of 10μM dexamethasone were given every 12 hours to the dexamethasone induced groups and IP injections of PBS given to the control groups. To ensure that the dexamethasone injections were inducing tTGase within the tumours, hamsters were injected with PBS or 10μM dexamethasone every 12 hours for 48 hours. After 4 injections of dexamethasone or PBS, the tumours harvested, homogenised in homogenising buffer (detailed in the Materials & Methods Chapter) and assayed using the tTGase assay detailed in the Materials & Methods Chapter. The data shown in Figure 3.18 indicates a 4.5-fold increase in tTGase activity in tumours treated with dexamethasone.

Figure 3.18: Dexamethasone induction of tTGase within the Met B fibrosarcoma



Met B tumours were grown to a diameter of approx. 0.5cm. Intraperitoneal injections of either PBS (control) or 10μM dexamethasone were carried out every 12 hours for 48 hours. The tumours were harvested and assayed for tTGase as described in the Methods Chapter. Data expressed as a mean of 3 tumours ± S.E.M. The results show a significant increase in tTGase in response to dexamethasone treatment ($p \leq 0.05$).

3.2.7.2 Induction of transglutaminase-associated cell death within the Met B tumour

When carrying out pharmacological studies on tumours, it is beneficial to give systemic injections for a number of reasons:

- Intratumour injections will cause a varying amount of "needle damage" with each injection. This damage cannot really be quantified as the extent of the damage will be dependent on a number of factors:
    - The travelling of the needle through the tumour tissue.
    - The pressure of the liquid coming out of the needle will vary slightly with each injection.
    - Intratumour injections are carried out while slowly removing the needle to provide a space in the tumour tissue in which the injected material can be pushed into before diffusing into the surrounding tissues. The speed of withdrawal and therefore the pressure exerted on the tumour will differ with each injection.
- If the treatment works, a systemic injection will reach all tumour cells in the body.

Due to dexamethasone being relatively non-toxic, it was decided to inject this intraperitoneally (IP). Ionomycin however, is a non-tumour specific ionophore. This needs to be delivered to the site of action as this makes it a very toxic substance. For this reason, it was decided to inject ionomycin directly into the tumour.

The tumours were grown to about 0.5cm in diameter. The hamsters were split into four groups:

- Control group with intratumour injections of DMSO
- Control group with intratumour injections of ionomycin
- Dexamethasone induced group with intratumour injections of DMSO
- Dexamethasone induced group with intratumour injections of ionomycin

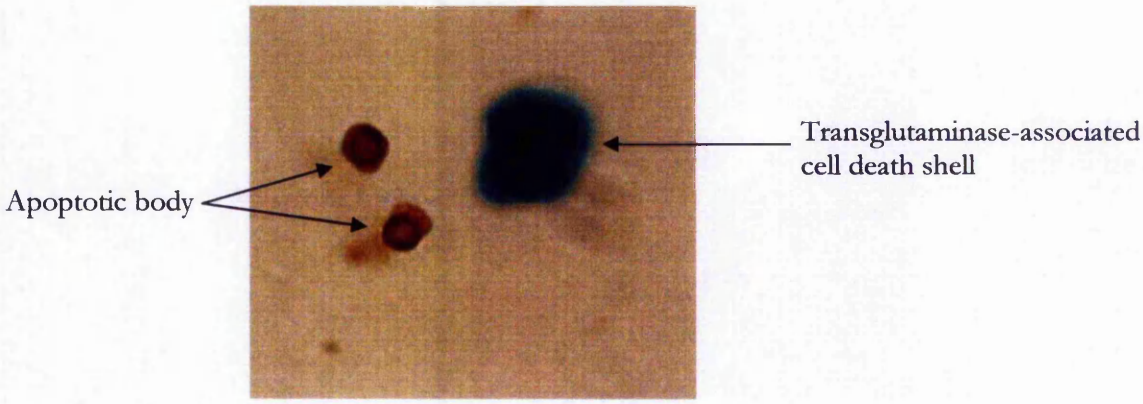
PBS/ Dexamethasone was injected intraperitoneally every 12 hours for 48 hours prior to, and throughout the intratumour injections. DMSO and ionomycin was injected directly into the tumour. The tumour was harvested from the animal after 16 hours. The tumour was weighed, measured and treated with SDS to isolate the insoluble shells (as described in the Methods Chapter) and stained with the FraGeL DNA fragmentation kit (Calbiochem). No differences in tumour sizes or weights were observed between the groups, although the short time course of the experiment would account for this. The FraGeL kit stains the Klenow-fragment of DNA left at the end of DNA digested during apoptosis. The counterstain used is Methyl Green, which stains intact DNA. The transglutaminase-associated cell death shells stained green and the apoptotic bodies stained brown (Figure 3.19a). The number of shells and apoptotic bodies were counted in three fields of view for each of the tumour homogenates. The number of each was normalised by counting the number per mg of tumour tissue (Figure 3.19b). The results show an overall higher number of apoptotic bodies than shells in all samples. However, in the tumours treated with both dexamethasone and ionomycin, there was a statistically significant increase in the number of shells and a substantial decrease in the number of apoptotic bodies when compared with untreated tumours, or tumours treated with just one agent. The liver and kidneys were also removed and treated in the same way to try and establish if

this method of cell death occurred naturally. Shells were isolated from both organs, but to a greater extent in the kidneys (Figure 3.20).

Figure 3.19: (a) Picture of "apoptotic body" and "transglutaminase-associated cell death shell" when stained with FraGeL DNA fragmentation kit (Calbiochem) & (b) Relative amounts of SDS-insoluble material isolated from Met B tumour

*Met B tumours were grown in Golden Syrian hamsters to a size of 0.5cm in diameter. Animals were injected with either PBS or 10μM dexamethasone every 12 hours for 48 hours. Intratumour injections of DMSO or ionomycin were given every 12 hours for 36 hours. Tumours were harvested, weighed and SDS-insoluble material obtained as described in the Methods chapter. This was air-dried onto a glass slide and stained using the FraGeL kit (Calbiochem), (a). The different coloured shells were counted in each sample and normalised by giving the amount of material per mg of tissue. Data expressed as a mean of 6 tumours ± SEM , (b). *'s show significant differences when compared with the Met B group for respective SDS-insoluble material ($p \leq 0.05$).*

(a)


Apoptotic body
Transglutaminase-associated cell death shell

(b)

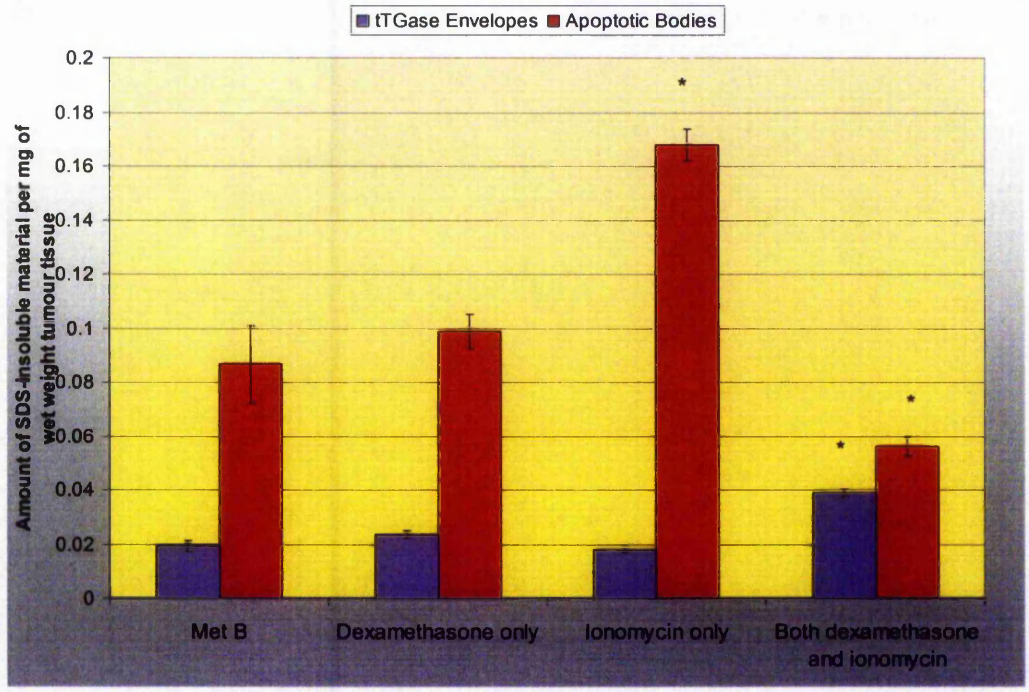
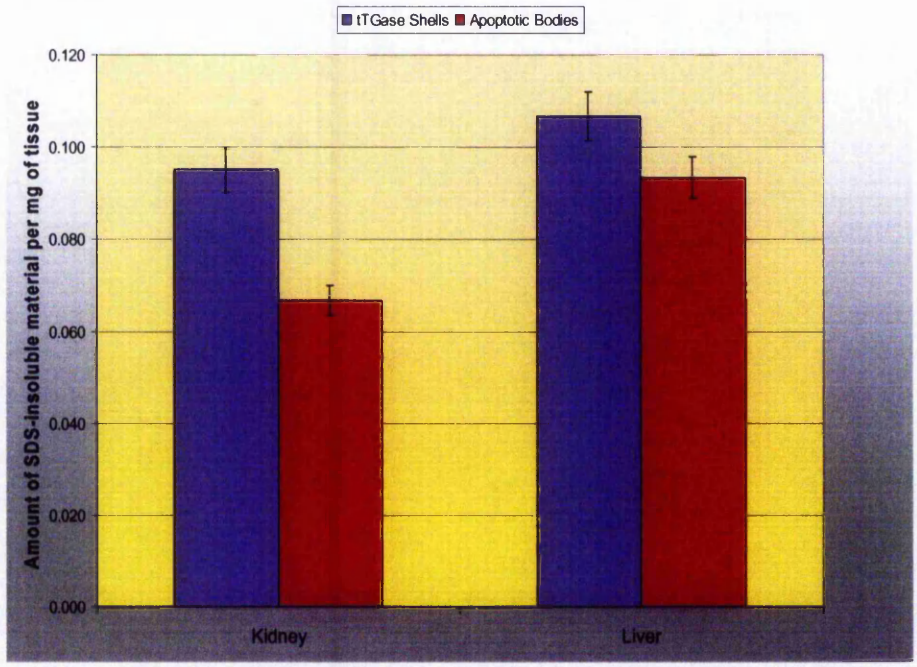

tTGase Envelopes
Apoptotic Bodies
Amount of SDS-insoluble material per mg of wet weight tumour tissue
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Met B
Dexamethasone only
Ionomycin only
Both dexamethasone and ionomycin

Figure 3.20: Relative amounts of SDS-insoluble material isolated from hamster liver and kidneys



Liver and kidneys were removed from Golden Syrian Hamsters, weighed and the SDS-insoluble material obtained as described in the Materials & Methods Chapter. This was air-dried onto a glass slide and stained using the FraGeL kit (Calbiochem). The different coloured shells were counted in each sample and normalised by giving the amount of material per mg of tissue. Data expressed as mean of organs from 6 animals ± SEM.

3.3 Discussion

These studies have shown that dexamethasone can induce tTGase in a dose-dependent manner in the Met B fibrosarcoma cells. This induction takes place at a genetic level, rather than at a post-translational modification stage, as seen in other cell types (Goldman, 1987, Fukuda *et al.*, 1994).

tTGase has been implicated in the formation of detergent-insoluble bodies, previously reported as apoptotic envelopes, found in cells undergoing programmed cell death (Fesus *et al.*, 1987, Knight *et al.*, 1991), which have now been isolated from a number of cell lines (Marvin *et al.*, 1992).

Dexamethasone treatment of Met B cells has been shown to give a 329% increase in apoptosis, as measured by the formation of SDS-insoluble bodies (Johnson, 1995). However, this is an old method of apoptosis detection, that has rarely been detected *in vivo*, so it was decided to investigate this further, using more up to date methods. When the experiment was carried out using the ApoTag method of apoptosis detection, which measures DNA-fragmentation, the results showed no increase in "classical" apoptosis. The experiment was also repeated using the ApoAlert kit (which measures the levels of active caspase-3, activated as part of the apoptotic pathway) and ApoGlow kit (which measures the ratio of ADP:ATP in the cell). Using all of these methods, no increase in apoptosis was detected. If anything, in all cases, there appeared to be a slight protective effect from the dexamethasone, but this was only statistically significant when measuring the induction of caspase-3. It is also interesting to note, that the ApoGlow assay measures ATP levels as an indicator of cell viability. The viability of cells also appeared to be unaffected by dexamethasone treatment. This may have been due to the relatively small number of cells undergoing apoptosis in this experiment. This was reinforced by the formation of shells in clones expressing high levels of bcl-2, a powerful apoptosis inhibitor.

Once it was established that the formation of these "apoptotic bodies" was not the result of "classical" apoptosis, it was decided to investigate their presence, and more importantly, the reason why they were increasing with dexamethasone treatment. It was postulated that the cells were probably being damaged during the processing steps of the experiment, leading to the formation of the envelope as tTGase was activated by the influx of Ca^{2+}, which in turn crosslinks all available substrates. Cells were harvested and immediately incubated with a fluorescently labelled tTGase primary amine substrate (fluorescein-cadaverine). Covalent incorporation of the labelled amine would give an indication of tTGase activity during the processing steps. When viewed under a fluorescent microscope, the dexamethasone treated cells showed a 3-fold increase in incorporation of the fluorescent substrate compared to the non treated cells. This correlates well with the 329% increase in "apoptotic bodies" observed in the initial experiments and the increase in tTGase activity observed after dexamethasone treatment. This set of experiments showed the discovery of a new form of cell death that was a sort of in-between the rigidly planned and executed apoptosis and the uncoordinated necrosis. The result of this form of cell death is "ghosts" or "shells" of slightly smaller size than normal cells. In the case of these set of experiments, this was probably brought about by the increase in intracellular tTGase, which under normal physiological conditions, the cross-linking action of the enzyme is inhibited by the relatively high levels of GTP and low [Ca^{2+}] (Smethurst and Griffin, 1996). However, treatment during the experiment, probably due to the action of trypsin during the cellular harvesting, causes small holes to form in the cell membrane. This in turn leads to an influx of Ca^{2+} and thus activation of the cross-linking activity of tTGase. tTGase is a very promiscuous enzyme and as such cross-links intracellular proteins, forming the shells observed (Chowdhury *et al.*, 1997, Kang *et al.*, 1995, Cohen and Anderson, 1987, Puszkin and Raghuraman, 1985). This theory was substantiated when treatment with dexamethasone and ionomycin gave a reduction in apoptosis. Cells treated with dexamethasone and a number of

known apoptotic inducing reagents showed roughly similar levels of classical apoptosis. However, cells treated with just ionomycin showed high levels of apoptosis. Cells pre-treated with dexamethasone showed a much-reduced level of apoptosis. It is postulated that the Ca^{2+} ionophore causes a calcium influx into the cell, thus activating the elevated levels of intracellular tTGase, causing the cross-linking of intracellular proteins and forming the shells. This happens much more quickly than the apoptotic pathway, and so the "protective" effect observed is the cell dying in a different, more rapid manner, but with minimum leakage of cellular DNA. This experiment is complicated by the fact that dexamethasone can have a protective effect from apoptosis by induction of Bcl-2 (Gorman *et al.*, 2000). However, this is unlikely to be the case as apoptosis was not inhibited by dexamethasone addition in the actinomycin D samples.

To see if this death could be induced as a potential treatment of tumours, Met B tumours were grown in hamsters and the tumours treated with dexamethasone and ionomycin. The SDS-insoluble material isolated from the experiments showed both apoptotic and transglutaminase-associated cell death products. The larger, almost cell-sized bodies described in earlier studies (Fesus *et al.*, 1987, Knight *et al.*, 1991, Melino *et al.*, 1994), contained intact DNA, not the fragmented DNA found in apoptotic cells. This was thought to be the product of transglutaminase-associated cell death. Smaller, highly crosslinked envelopes were also found in the samples. These stained for fragmented DNA, and are the apoptotic bodies formed at the terminal stages of the apoptosis pathway. tTGase may still be involved in the formation of these apoptotic bodies as there is still considerable evidence of tTGase gene induction in response to apoptotic stimuli (Goldman, 1987, Fesus, 1993, Fesus *et al.*, 1996, Melino and Piacentini, 1998). However, the SDS-insoluble material isolated in these sets of experiment demonstrated that both types of cell death can be induced within tumours. It also showed a much higher percentage of apoptotic bodies than shells in most tumours, but

this is to be expected as one cell dying by apoptosis will produce several apoptotic bodies due to membrane blebbing. However, a cell dying by tTGase-associated cell death should only produce one shell, as the cell is effectively "fixed" by the cross-linking of the tTGase.

The liver and kidneys of some of the control animals were also harvested to see if evidence of tTGase-associated cell death could be observed in a "normal" animal. The liver and kidneys were chosen as high levels of tTGase have been shown in these tissues. The results showed a surprisingly high number of shells compared to apoptotic bodies in these normal tissues. This may be due to the body clearing away the apoptotic debris very quickly as it is formed. This type of cell death has since been implicated in kidney fibrosis leading to end stage renal failure (Johnson *et al.*, 1997, Johnson *et al.*, 1999).

Chapter 4

TISSUE TRANSGLUTAMINASE IN THE P8 RAT OSTEOSARCOMA SOLID TUMOUR

4.1 Introduction

The involvement of tissue transglutaminase (tTGase) in tumour growth has been an area of contention for some years. The enzyme has been implicated in many aspects of tumour growth and progression largely due to its implied role in a wide range of cellular functions, such as cell death (Knight *et al.*, 1991), cell growth (Mian *et al.*, 1995), cell migration (Balklava *et al.*, 2002), cell adhesion (Belkin *et al.*, 2001) and ECM stabilisation (Kong and Korthuis, 1997). There has been much evidence suggesting that the levels of tTGase are reduced in the neoplastic when compared to normal tissue (Hand *et al.*, 1988), but this has been challenged in recent years by researchers stating that the tTGase levels in the tumour tissue can alter in different tumours and depends on the particular type of tumour (Takaku *et al.*, 1995), although this could also depend on the stage of tumour growth at which the measurement is made. It has been hypothesised by several researchers that the extracellular matrix stabilising role of tTGase (described in various "normal" cell types (Jones *et al.*, 1997, Gaudry *et al.*, 1999, Martinez *et al.*, 1994)) may be reduced in the some primary tumours to assist tumour escape (McCarthy *et al.*, 1986, Hager *et al.*, 1997) and then increased in the secondary metastatic tissue in order to facilitate tumour colonisation of the new tissue.

The aim of this research was to investigate the way in which tTGase levels may affect tumour growth and progression in the partially-characterised, metastasising rat osteosarcoma tumour model (P8). The reason for changing tumour model was to be able to carry out experimental metastases experiments. This would have been impossible with the Met B tumour as it is of hamster origin and hamsters have no tail in which to inject the tumour cells to induce experimental metastases.

Previous studies using this osteosarcoma have shown a reduction and shift in sub-cellular localisation of tTGase during tumour growth (Barnes *et al.*, 1985) and a corresponding with a reduction in polyamine content of the tumour (Hand *et al.*, 1987) during tumour metastasis.

4.2 Results

4.2.1 Tissue Transglutaminase in P8 Tumour Development

Tumours were grown in AS rats and removed at various stages of tumour growth. The tTGase activity of these primary and secondary tumours was assayed to investigate the functionality of the enzyme in each tumour sample during tumour growth and progression. It is hypothesised that tTGase levels and/or activity must drop below a certain level, thus reducing the stability of the ECM, facilitating cell migration and thus promoting tumour invasion and metastasis. In order to investigate this, tumours were harvested at different stages of their growth. The tumour was then homogenised and assayed for tTGase activity using the [^{14}C]-putrescine incorporation into N,N'-dimethylcasein. Figure 4.1 shows a reduction in tTGase activity during tumour growth. The dotted line on the graph illustrates the hypothesised point of metastasis. This was deduced by resecting the primary tumour at different stages of development and allowing any secondary metastatic tumours to propagate in the lungs. The results suggest that the tumour must grow to a size of approximately 900mm^3, with a tTGase activity reduced to about 0.145 units/mg of protein in order to facilitate metastasis. Post metastasis, the tTGase activity of the primary tumour continued to drop to a level of 0.004 units/mg of protein.

Table 4.1 shows the tTGase activity in some of the primary tumours displayed in Figure 4.1 and their secondary tumours isolated from the lungs. The whole of the secondary tumour was usually used in the assay, as the samples were so small. Interestingly, tTGase activities of the secondary tumours were much higher than that of the primary tumours, showing a 10-fold increase from the hypothesised level of metastasis to the levels observed in the secondary tumours.

4.2.2 Analysis of tTGase in tumour homogenates by western blotting

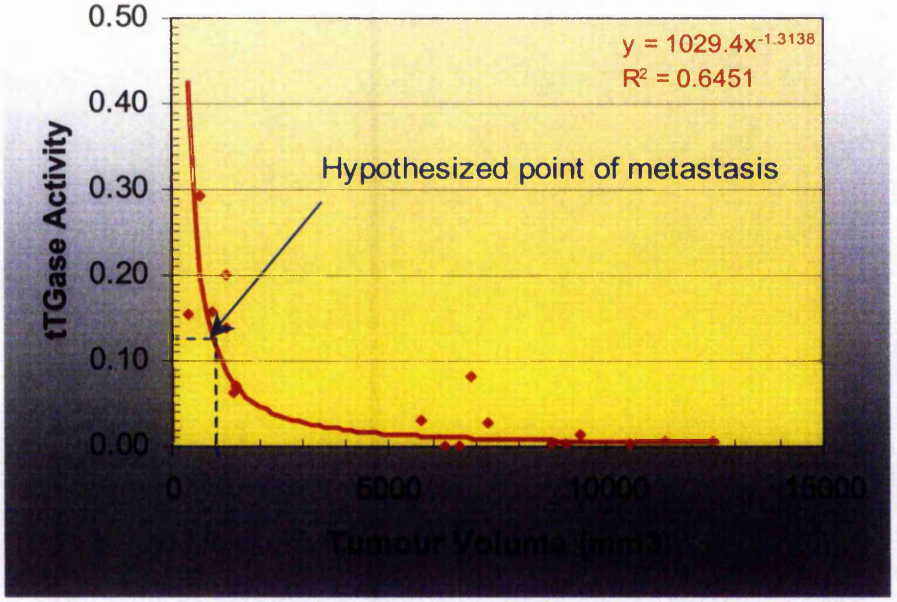
Tumour homogentates obtained from tumours harvested at different stages of tumour growth were analysed by PAGE using 10% SDS-PAGE gels. The loadings were corrected for both protein and DNA, in order to give a normalised result for both protein and cell number. In both cases the level of tTGase detected

decreased as the tumour grew, but increased again in the metastatic tumour compared to the primary tumour (Figure 4.2).

4.2.3 P8 tumour morphology

To investigate any changes in tumour morphology during tumour progression, tumour sections embedded in paraffin wax were stained with Gills haematoxylin & eosin (Figure 4.3). As the tumour progressed, the morphology of the cells within the tumour changed from a fibroblast-like (Figure 4.3d; Smaller cells with few non-staining areas between cells – i.e. little ECM deposition) to a more osteoblast like appearance (Figure 4.3f; Larger cells with vast non-staining areas between cells – i.e. large amounts of ECM). The heterogeneous population of cells in different stages of differentiation was observed in middle-aged and old tumours (Figures 4.3b,c).

Figure 4.1: Graph to show changes in tTGase activity in a growing tumour.



Small pieces of P8 tumour of approximately equal size were sub-cutaneously implanted into the flank of AS rats. The point of metastasis was investigated by resecting tumours at different stages of tumour development. The dotted blue line indicates the approximate point in tumour growth that metastasis occurs. tTGase activity is expressed in Units / g wet weight tissue (n=18).

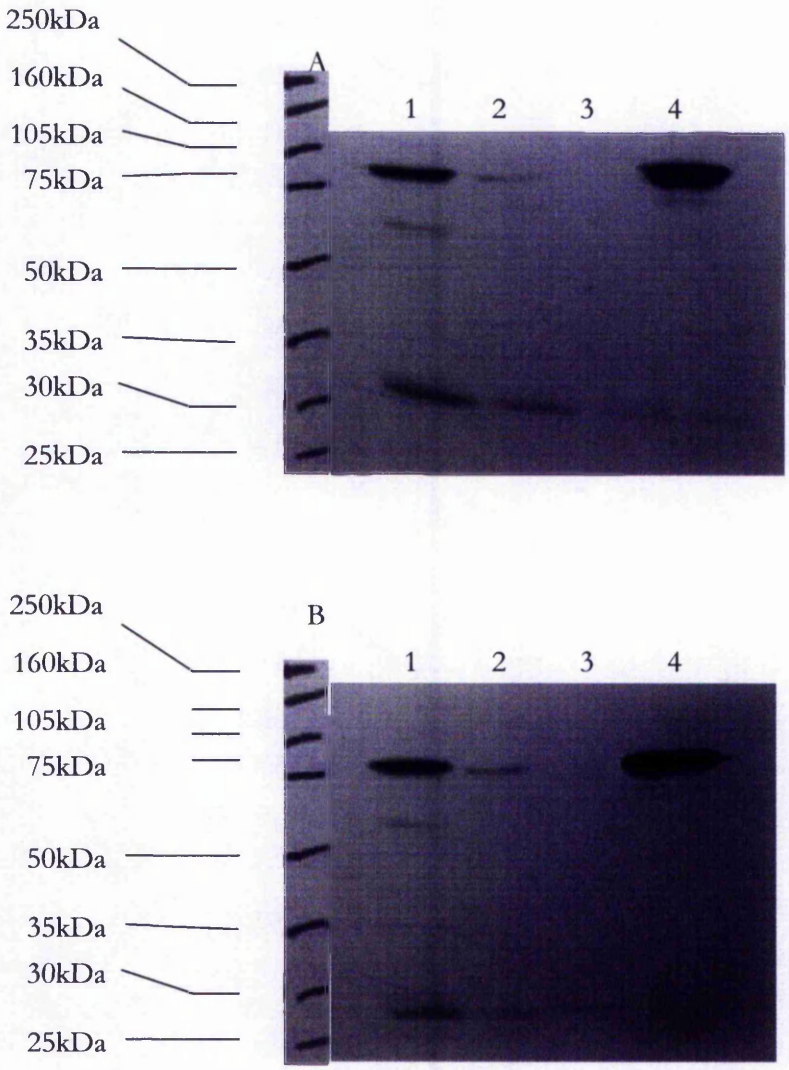
Table 4.1: tTGase activity of primary tumours during various stages of development & their secondary tumours.

Tumour Size (mm^3)	tTGase Activity (Units / g wet weight tissue)
672	0.291
1237	0.2
7290	0.026
8736	0.004
9408	0.013
10560	0.004
11408	0.006
Met 1	1.158
Met 2	1.573
Met 3	1.546

Small pieces of P8 tumour of approximately equal size were sub-cutaneously implanted into the flank of AS rats. Metastases were obtained by resecting tumours at different stages of tumour development and allowing the secondary tumours to grow. Secondary tumours are denoted by 'Met' prefix. . tTGase activity is expressed Units / g wet weight tissue (n=6).

Figure 4.2: Western blots of P8 tumour homogenates probed with the CUB 7402 α-tTGase antibody

Small pieces of P8 tumour were sub-cutaneously implanted into the flank of AS rats. Metastases were obtained by resecting tumours at different stages of tumour development and allowing the secondary tumours to grow. The tumours were harvested and homogenised as described in the Materials and Methods chapter. Homogenates were run on a 10% SDS-PAGE gel (Loadings normalised using protein [A] and DNA [B]) and blotted onto nitrocellulose. This was probed with CUB7402 α-tTGase antibody and revealed using an anti-mouse HRP conjugate, and developed using ECL for 1 min onto Kodak Biomax light film. Lane 1: Primary pre-metastatic tumour (approx. vol. = 672mm^3); Lane 2: Primary tumour about to undergo metastasis (approx. vol. = 1274mm^3); Lane 3: Primary post-metastatic tumour (approx. vol. = 10560mm^3): Lane 4: Secondary tumour.


250kDa
160kDa
105kDa
75kDa
50kDa
35kDa
30kDa
25kDa
A
1
2
3
4
250kDa
160kDa
105kDa
75kDa
50kDa
35kDa
30kDa
25kDa
B
1
2
3
4

4.2.4 Immunohistochemical analysis of tTGase distribution in the P8 tumour

The paraffin wax sections were also used for tTGase staining using the CUB7402 α-tTGase mAb. However, this staining did not work, as paraffin wax embedding often masks the antigen, thus making immunohistochemical analysis impossible. To alleviate this problem, the much less harsh method of cryostat sectioning was used to prepare samples for immunohistochemical analysis.

Cryostat sections of tumour at various stages of growth were stained with CUB 7402 α-tTGase mAb (Figure 4.4). This is likely to stain only the tTGase present in the extracellular matrix (ECM) as the majority of the cytosolic enzyme will be washed away in the buffer containing EDTA and protease inhibitors, during the preparatory stages of the procedure (See Section 4.2.4).

It was observed that the level of staining decreased as the primary tumour grew (Figures 4.4a-c), which mirrored the activity results (Figure 4.1). Staining of the metastatic tissue also reinforced the activity assay and Western blotting results as very intense staining was observed in the secondary tumour (Figure 4.4d).

The staining of tTGase in the primary tumour sections appeared to be in regular line patterns, possibly along extracellular fibrils (Figures 4.4a,b). As the tumour progresses, these lines seem to decrease in length and become less defined.

4.2.5 Fibronectin deposition in the P8 tumour

Cryostat sections stained for fibronectin showed a steady increase in fibronectin levels as the tumour grows (Figure 4.5). The metastatic secondary tumour tissue showed sparse fibronectin distribution throughout the tumour, which is in agreement with the early pre-metastatic tumour staining, as the secondary tumour has had less time to lay down much matrix.

In agreement with the H & E staining (Figure 4.3), the tissue showed areas of differing morphology in the later tumours when stained for tTGase and fibronectin (Figures 4.4 & 4.5), as observed by the reduction in size and intensity of nuclear staining.

4.2.6 Collagen deposition in the P8 tumour

The Massons Trichrome stain for general collagen was used on the paraffin wax sections of tumour to determine the general collagen distribution within the tumour. The level of collagen staining was fairly low throughout the tumour, with the majority of staining around the outside of the tumour in the connective tissue (Figure 4.6).

In all three stains (tTGase, fibronectin and the more general Massons Trichrome collagen stain), small areas of intense staining could be observed in fibrillar "lines" along the tumour section. This could indicate the incorporation of tTGase into the ECM. These lines appear to become shorter and less frequent as the tumour grows.

4.2.7 Angiogenesis in the P8 tumour

To investigate the level of angiogenesis within the tumour, sections were stained with an antibody raised against endothelial cell marker, Von Willebrand factor. This staining revealed little angiogenesis within the P8 tumour, with endothelial cells only being present around the edge of the larger tumours and in the lung tissue surrounding the secondary tumours (Figure 4.7).

Figure 4.3: Change in morphology of P8 tumour as it progresses.

Small pieces of tumour were sub-cutaneously implanted into the flank of AS rats. Tumours were removed, fixed in 4%(v/v) neutral buffered formalin, blocked in paraffin wax and sectioned prior to staining with Gills haematoxylin & eosin. (A) Primary pre-metastatic tumour (approx. vol. = 672mm3) x40; (B) Primary tumour about to undergo metastasis (approx. vol. = 1274mm3) x40; (C) Primary post-metastatic tumour (approx. vol. = 10560mm3) 40x; (D) Primary pre-metastatic tumour (approx. vol. = 672mm3) x160; (E) Primary tumour about to undergo metastasis (approx. vol. = 1274mm3) x160; (F) Primary post-metastatic tumour (approx. vol. = 10560mm3) x160. The black arrows illustrate areas of fibroblast-like cells within the tumour. The white arrows indicate areas of osteoblast-like cells within the tumour.

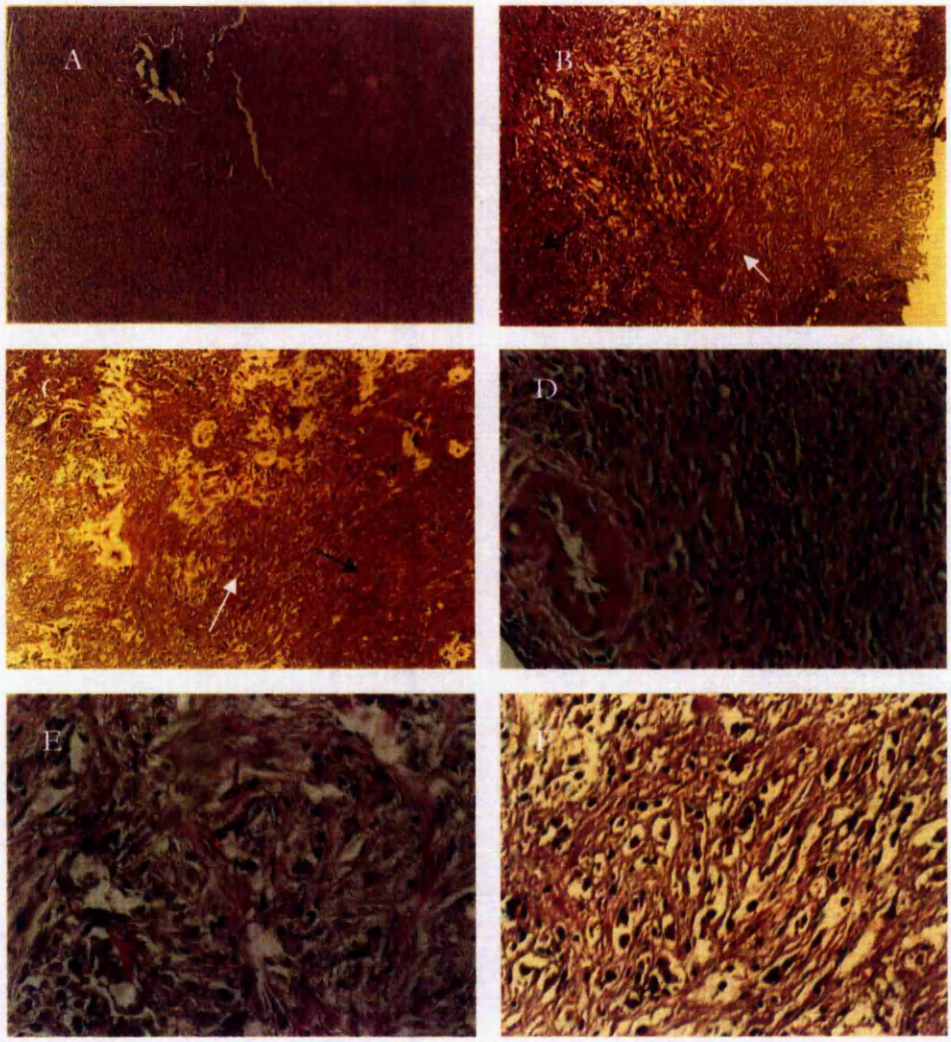

A
B
C
D
E
F

Figure 4.4: Changes in tTGase antigen as the tumour progresses.

Small pieces of P8 tumour were sub-cutaneously implanted into the flank of AS rats. Cryostat sections were stained with CUB7402 α-tTGase mAb (1/50 dilution) and fixed, prior to counter-staining with propidium iodide (5μg/ml) as described in the methods section. The metastases were propagated by resection of the primary tumour load. (A) Primary pre-metastatic tumour (approx. vol. = 672mm^3); (B) Primary tumour about to undergo metastasis (approx. vol. = 1274mm^3); (C) Primary post-metastatic tumour (approx. vol. = 10560mm^3): (D) Secondary tumour. Channels 1, 2 and 1 + 2 are shown for each section. Arrows show 'lines' of staining.

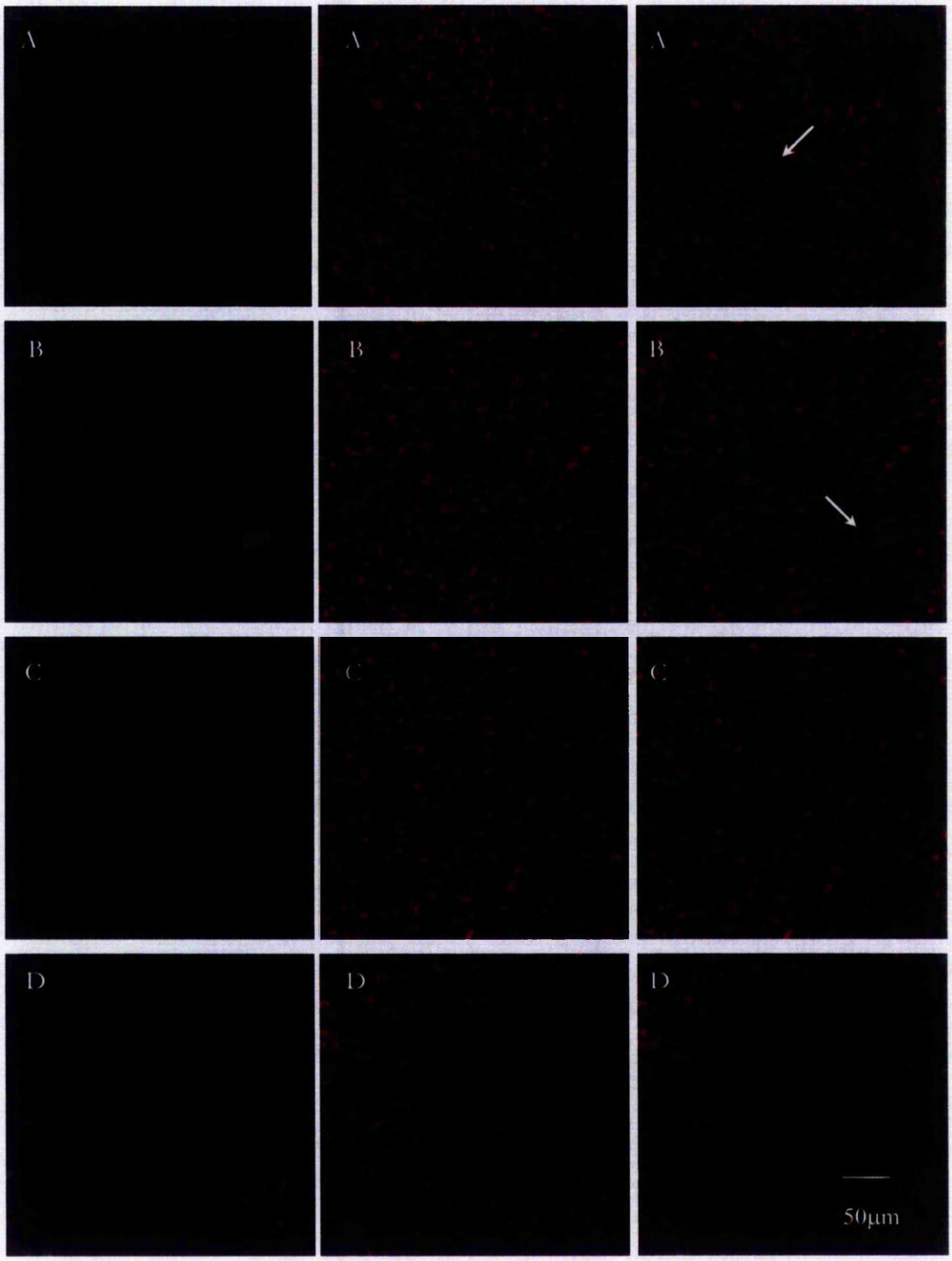

A
A
A
B
B
B
C
C
C
D
D
D
50μm

Figure 4.5: Changes in fibronectin deposition during P8 tumour growth

Small pieces of tumour were sub-cutaneously implanted into the flank of AS rats. Cryostat sections were stained with a-fibronectin mAb (1/100 dilution; Sigma) and fixed prior to counter-staining with propidium iodide (5μg/ml) as described in the methods section. The metastases were propagated by resection of the primary tumour load. (A) Primary pre-metastatic tumour (approx. vol. = 672mm^3); (B) Primary tumour about to undergo metastasis (approx. vol. = 1274mm^3); (C) Primary post-metastatic tumour (approx. vol. = 10560mm^3); (D) Secondary tumour. Channels 1, 2 and 1 + 2 are shown for each section.

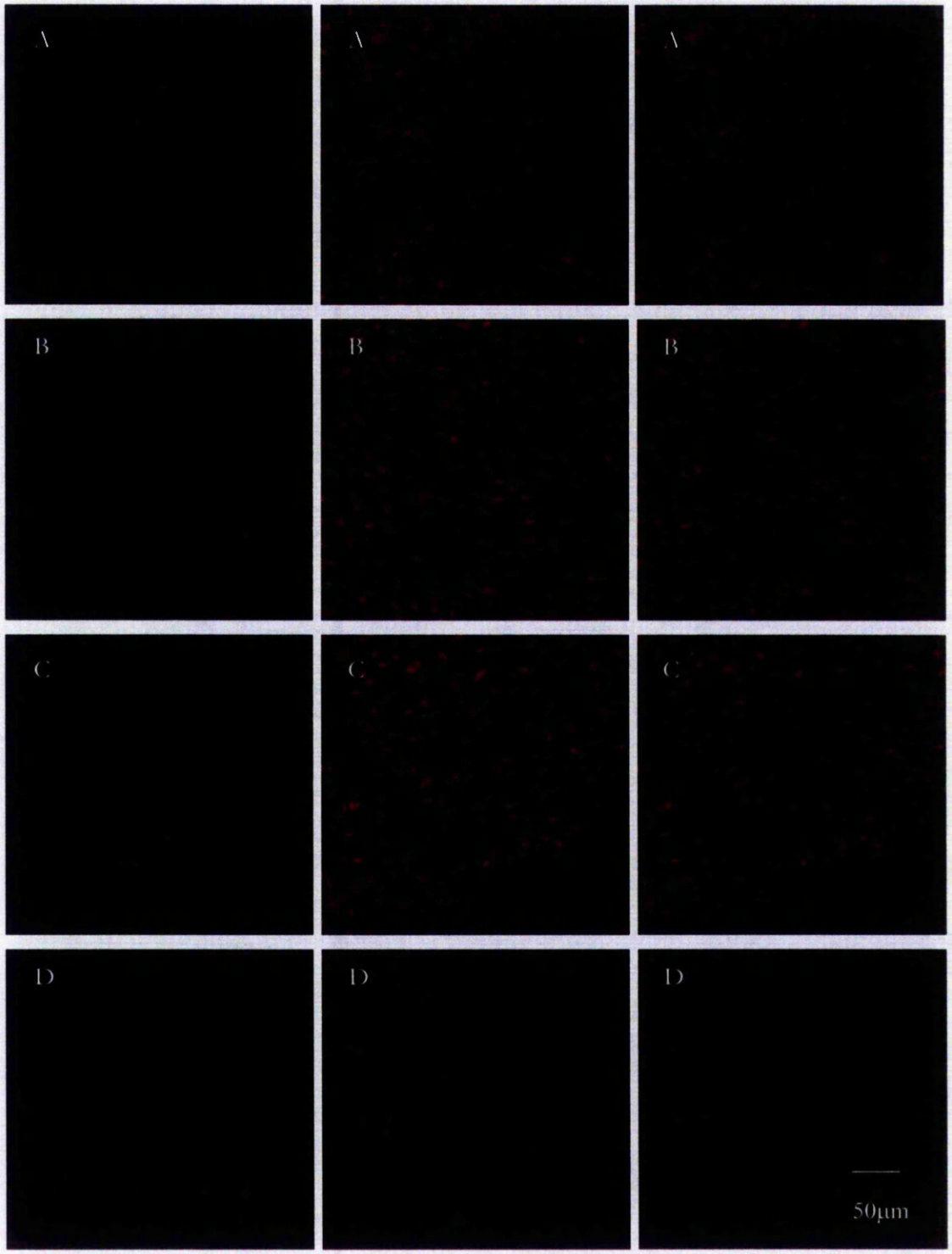

A
A
A
B
B
B
C
C
C
D
D
D
50μm

Figure 4.6: Massons trichrome stain for general collagen in P8 tumour

Small pieces of tumour were sub-cutaneously implanted into the flank of AS rats. Tumours were removed, fixed in 4%(v/v) neutral buffered formalin, blocked in paraffin wax and sectioned prior to staining with Massons Trichrome. (A) Primary pre-metastatic tumour (approx. vol. = 672mm^3) x20; (B) Primary tumour about to undergo metastasis (approx. vol. = 1274mm^3) x20; (C) Primary post-metastatic tumour (approx. vol. = 10560mm^3) x20; (D) Secondary metastatic tumour. The black arrows illustrate areas of collagen staining, white arrows highlight the changes in tumour morphology observed in the tumour.

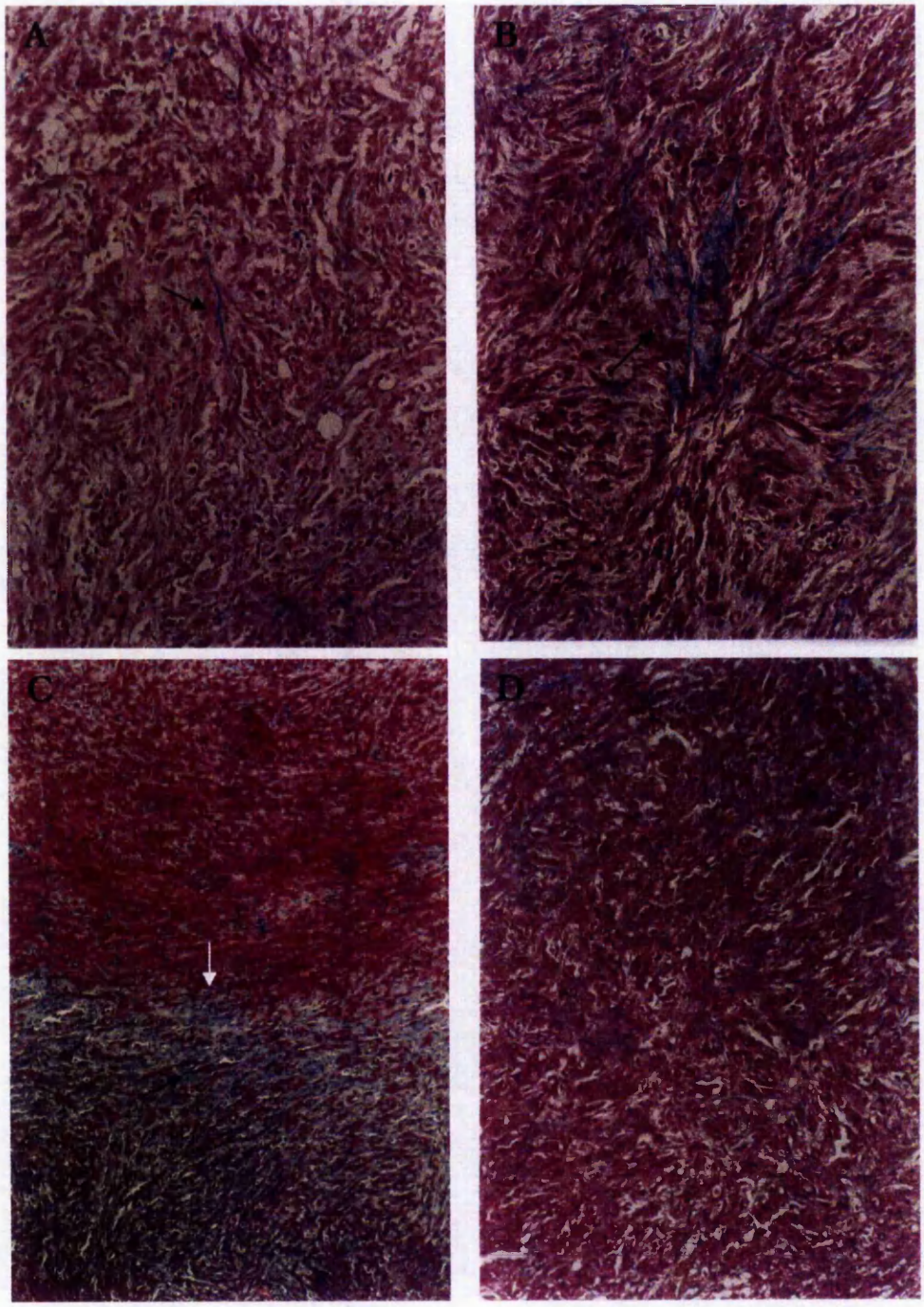
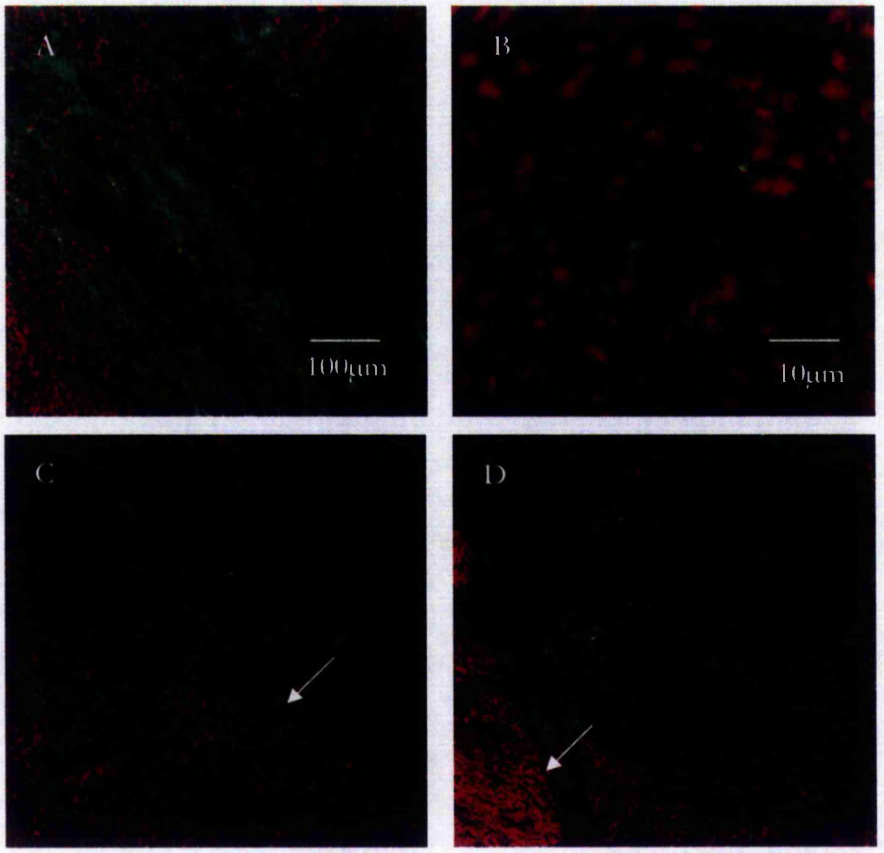

A
B
C
D

Figure 4.7: Angiogenesis in the P8 tumour



Small pieces of tumour were sub-cutaneously implanted into the flank of AS rats. Cryostat sections were stained with α-Von Willebrand factor polyclonal antibody (1/2000 dilution; Sigma) and fixed prior to counter-staining with propidium iodide (5µg/ml) as described in the methods section. The metastases were propagated by resection of the primary tumour load. (A) Likely longitudinal section of blood vessel in large tumour; (B) Likely transverse section of blood vessel in large tumour; (C) Blood vessels or bronchi in lungs next to secondary tumour; (D) Blood vessels or bronchi in lungs next to secondary tumour. The arrows indicate tumour / lung boundary.

4.2.8 In situ tTGase activity in the P8 tumour

In order to investigate the activity of the ECM bound tTGase enzyme in the P8 tumour, cryostat sections were incubated in the presence of the competitive primary amine substrate, fluorescein-cadaverine, such that this amine becomes incorporated into peptide bonds with γ-glutamyl residues in a tTGase-mediated mechanism. The resulting slides were viewed using a Leica confocal microscope (Figure 4.8). The level of incorporation of fluorescein-cadaverine into the section mirrored the tTGase activity assay and the staining results obtained for the tTGase antigen. One exception was in the very early tumour, where large areas of incorporation were observed. These did not stain for tTGase antigen and are postulated to be cells rich in other transglutaminases such as factor XIII or transglutaminase X. Individual cells incorporating large amounts of fluorescein-cadaverine were observed in the secondary tumour.

4.2.9 tTGase gene expression patterns in the P8 tumour measured by *in situ* hybridisation

The pattern of expression of the tTGase gene within the tumour was investigated using *in situ* hybridisation, using the method described in the Methods section. The expression of tTGase mRNA in the P8 tumour mirrored the results seen previously with the anti-tTGase antibody, with staining "lining" up in the tumour (Figure 4.9). Also observed were distinct areas of differing staining in the tumour, particularly around the time of metastasis. It is important to remember that this method of *in situ* hybridisation is qualitative, not quantitative, so no comparisons of the amount of mRNA present can be made. There were also "patches" of intense expression, mirroring the *in situ* activity results.

Figure 4.8: tTGase in situ activity in the P8 tumour

Small pieces of tumour were sub-cutaneously implanted into the flank of AS rats. Cryostat sections were incubated with incubated with FITC-Cadaverine (0.5mM, Molecular Probes) and fixed prior to counter-staining with propidium iodide (5μg/ml) as described in the methods section. The metastases were propagated by resection of the primary tumour load. (A) Primary pre-metastatic tumour (approx. vol. = 672mm^3); (B) Primary tumour about to undergo metastasis (approx. vol. = 1274mm^3); (C) Primary post-metastatic tumour (approx. vol. = 10560mm^3); (D) Secondary tumour; (E) Zoomed secondary tumour.

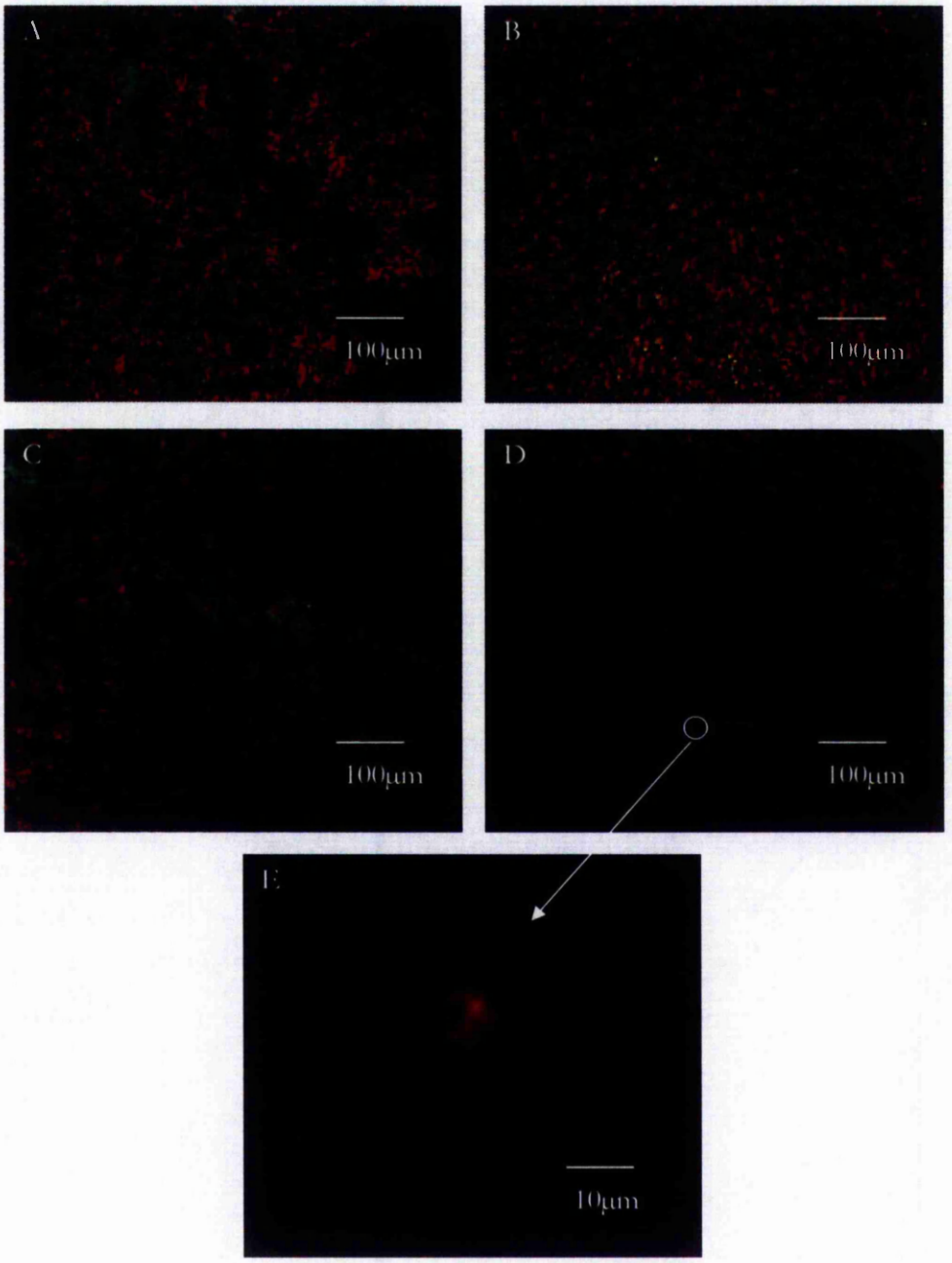
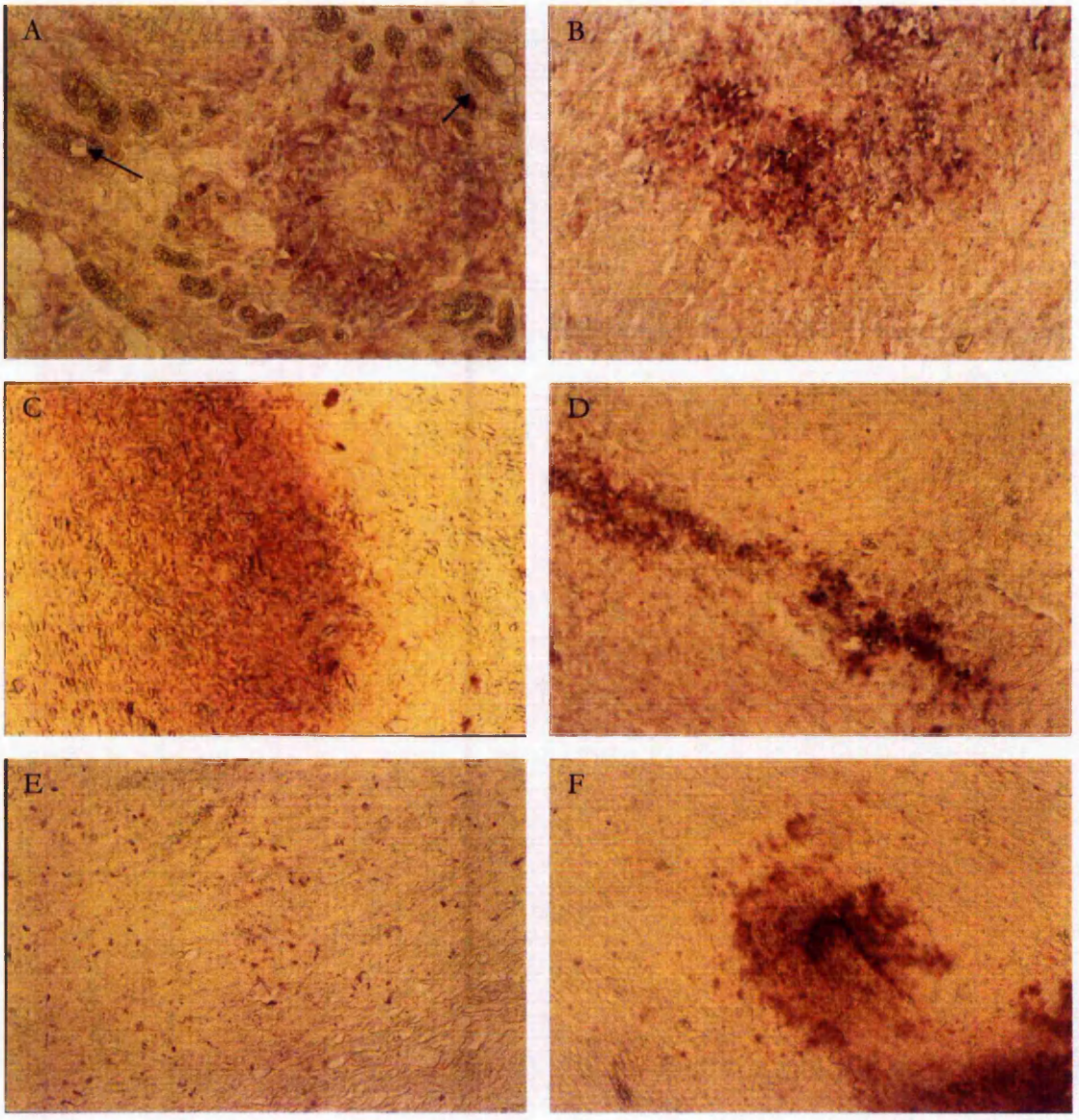

A
100μm
B
100μm
C
100μm
D
100μm
E
10μm

Figure 4.9: Patterns of tTGase mRNA expression in the P8 tumour

Small pieces of tumour were sub-cutaneously implanted into the flank of AS rats. Tumours were removed, fixed in 4%(v/v) neutral buffered formalin, blocked in paraffin wax and sectioned. Sections were hybridised with digoxigenin with antisense mRNA for tTGase. The digoxigenin label was revealed using an alkaline phosphatase detection system. (A) Primary pre-metastatic tumour (approx. vol. = 672mm^3) x40; (B) Primary pre-metastatic tumour (approx. vol. = 672mm^3) x40; (C) Primary tumour about to undergo metastasis (approx. vol. = 1274mm^3) x40; (D) Primary tumour about to undergo metastasis (approx. vol. = 1274mm^3) x40; (E) Primary post-metastatic tumour (approx. vol. = 10560mm^3) 40x; (F) Secondary tumour x40. The black arrows illustrate areas of similar staining to the bodies observed in the in situ activity assay, thought to be cells containing high amounts of other transglutaminases such as factor XIII or transglutaminase X, observed only in the early stages of tumour growth.


A
B
C
D
E
F

4.3 Discussion

These studies have shown that tTGase activity and antigen becomes reduced in the P8 osteosarcoma as the tumour progresses towards metastasis. Interestingly, the secondary tumours themselves have high levels of tTGase. This drop in activity has been thought to correlate with the tumours ability to metastasise (Barnes *et al.*, 1985, McCarthy *et al.*, 1986, Hager *et al.*, 1997). To investigate this further, resection studies were carried out showing that the P8 osteosarcoma metastases will metastasise shortly before the tumour grows to a size of 1000mm^3 and a tTGase activity of 0.13 nmol putrescine incorporated / hour / g wet weight tissue (Figure 4.1). Similar trends were also observed when tTGase activity was calculated using protein and DNA (results not shown).

This drop in activity is accompanied by a drop in antigen when measured by both western blotting and immunohistochemical staining. The immunohistochemical staining of the tumour sections also revealed small foci of cells that produce large amounts of extracellular transglutaminase. These lines of cells appeared to get shorter and less frequent as the tumour developed, perhaps suggesting a level of heterogeneity with the tumour. This could also indicate a destabilisation of the matrix of the tumour, to allow for the initial stage of metastases. This trend appeared to be made at a molecular level, as *in situ* hybridisation studies also revealed a reduction in staining and in the patterns of staining. It has been hypothesised that the extracellular tTGase is not active, but binds itself into the ECM via its affinity with fibronectin (Akimov and Belkin, 2001). To investigate this, the sections were incubated with a tTGase competitive primary amine substrate (fluorescein cadaverine), as this would only be crosslinked by extracellular bound tTGase, as the intracellular tTGase would be washed away during the processing of the slide. The patterns of incorporation observed and were found to be

similar to the tTGase antigen staining and mRNA distribution, suggesting that the extracellular enzyme maintains its transamidating ability.

Also coinciding with this drop in tTGase is a change in tumour morphology from a fibroblast-like to a larger, more rounded osteoblast-like state.

The change in morphology is accompanied by increased deposition of ECM, in particular collagen. This is not unexpected as normal osteoblasts lay down fairly large amounts of collagen and fibronectin during normal development and differentiation (Aeschlimann *et al.*, 1996).

The accumulation of structured ECM observed in the later samples suggests that matrix degradation is less than that observed in other tumours (Sugiura and Berditchevski, 1999, Benbow *et al.*, 1999, Westermarck and Kahari, 1999), as both the fibronectin and collagen staining appeared to be maintained throughout tumour progression.

There was much less angiogenesis than expected observed in the tumour, with the von Willebrand antibody only staining the endothelial cells in the surrounding lung tissue of the metastatic tumours and the very occasional blood vessel observed in the very large tumours. This may be due to the fact that the tumour has become cartilage-like. Cartilage is naturally in a low [O_2] environment. The O_2 and nutrients needed for cartilage naturally are carried from the synovial fluid, through the ECM (Buckwalter *et al.*, 1990), thus the cells in the P8 tumour may require less O_2 and nutrients than other cell types, negating the need for large scale angiogenesis within the tumour mass. It should be noted however, that the tumours were often highly vascularised around the outside of the tumour mass. This may suggest that the secondary tumours originate from the outer edge of the primary tumour as many researchers consider angiogenesis as the first step in the chain of events leading to metastasis (Bikfalvi, 1995).

Investigations into secondary tumour formation showed that the P8 rat osteosarcoma preferentially metastasises to the lungs, as no other secondary tumours were discovered at other locations. This is not uncommon in many tumour types (Engebraaten and Fodstad, 1999, Wolff *et al.*, 1998, Ju *et al.*, 1996, Zoller, 1995).

Chapter 5

USING THE P8 OSTEOSARCOMA AS A CELL CULTURE MODEL OF TUMOUR GROWTH

5.1 Introduction

Cell culture models of tumour growth have become well established in the field of cancer research (Beidler *et al.*, 1973, Ciccarone *et al.*, 1989). To further investigate the role of tTGase in tumour progression and metastases, it was decided to isolate cells from the solid primary tumour in order to characterise the behaviour of the cells in the more controllable *in vitro* environment. Previous work on other cell lines suggested that tumour cells transfected with tTGase cDNA under the control of a viral promoter blocks tumour growth (Johnson *et al.*, 1994). Given the reduction in tTGase expression in the solid P8 tumour, the idea to isolate cells from the solid tumour and produce high expressing tTGase clones was an obvious one. This chapter focuses on the characterisation of these cells to investigate the findings of (Johnson *et al.*, 1994) in a different cell / tumour model.

5.2 Results

5.2.1 Isolation of cells from the P8 tumour

Tumours were grown sub-cutaneously on the flank of AS rats as described in Section 2.2.3.1. At between 0.5 and 0.75cm in diameter, these tumours were harvested and the surrounding tissue cut away. Tumours of this size were used since they are of a reasonable size to work with and little necrosis within the tumour was observed. Once cleaned of connective tissue, the tumours were finely chopped using a scalpel in cell dispersion solution containing collagenase (See Section 2.2.3.8). Once the main bulk of the tumour mass has been dispersed, the cell solution was incubated at 37°C for 1 hour. The cells were then washed in DMEM containing 10% FCS before being seeded into a $75cm^2$ tissue culture flask. Cells were then passaged at least 5 times to remove the other non-tumour cells contaminating the culture. Figure 5.1 shows photographs of P8 cells in culture.

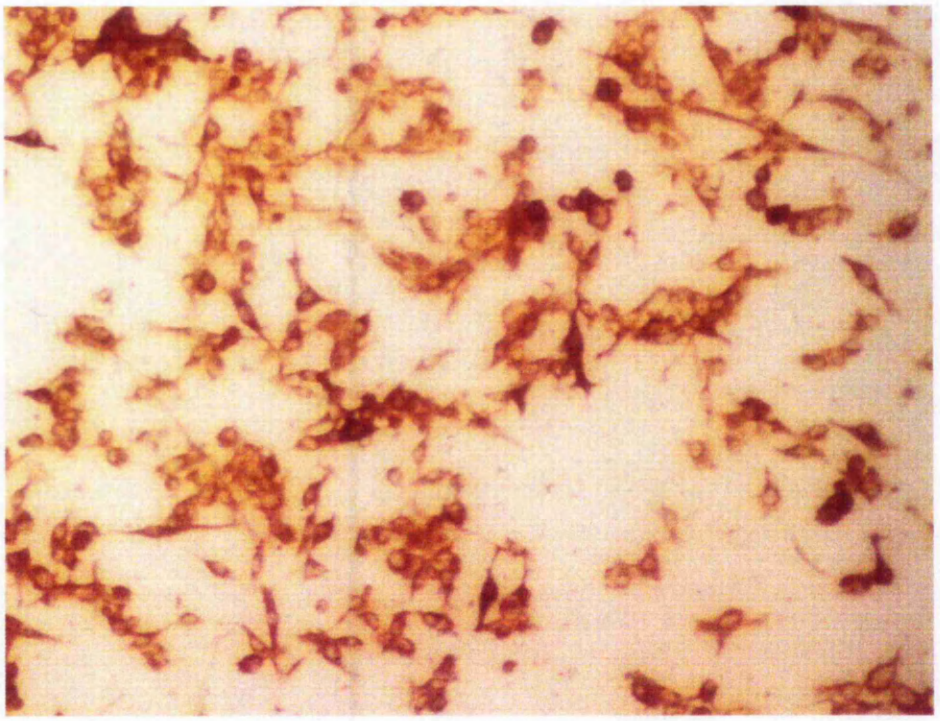
5.2.2 Growth Characteristics of the P8 cell line

Studies to investigate the growth rate of the cell line were carried out. 10^4 cells were seeded into each of 12 T$75cm^2$ tissue culture flasks and incubated at 37°C, 5%CO_2. At 24 hour time-points, three flasks were removed and the cells from each counted as described in Section 2.2.1. After 96 hours, the results were plotted and the doubling time of the cells was found to be approx 14.5 hours (Figure 5.2).

5.2.3 tTGase activity in the P8 cell line

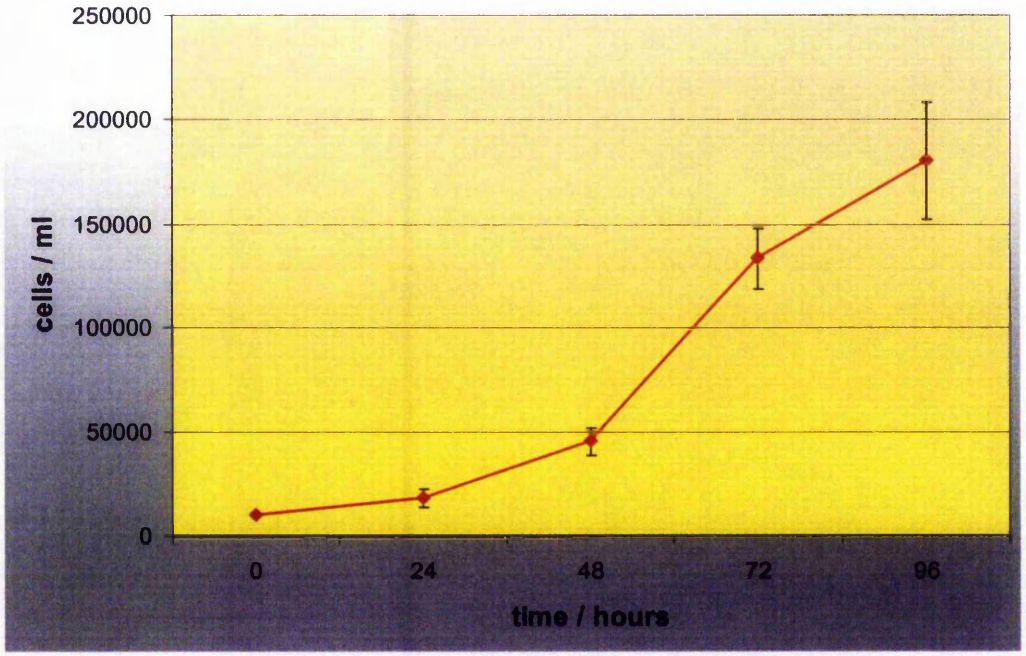
In order to assess the similarities between the cell culture and the solid tumour models, tTGase assays were carried out on sub-confluent and confluent cells. The results showed a non-significant reduction in activity as the cells became confluent (Figure 5.3), as such, wherever possible, future experiments were carried out when the cells were approx 70% confluent.

Figure 5.1: Pictures of P8 osteosarcoma cells in culture after being isolated from solid tumour and stained with CUB7402 mAb raised against tTGase



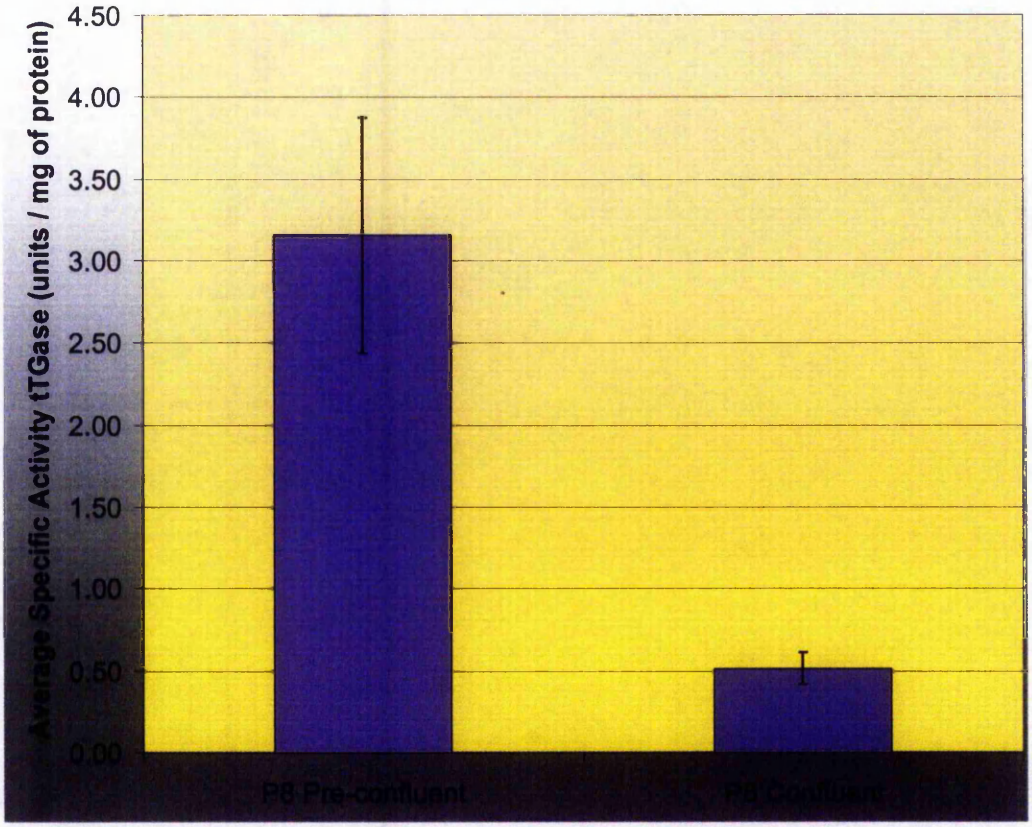
Cells were isolated from P8 solid tumour as described in the Materials and Methods Section. Cells were passaged 5 times to remove any non-transformed / tumour cells. The cells were grown in a 96-well plate for 36 hours. The culture medium was removed and the cells carefully washed twice in Dulbeccos PBS (1x). The cells were fixed with 4% neutral buffered formalin and stained with CUB7402 mAb as described in the Materials and Methods Section. The photo was taken using an Olympus DP10 microscope digital camera attached to an Olympus CK2 light inverted microscope.

Figure 5.2: Growth curve of the P8 cell line



10^4 Cells were seeded into 12 T75 tissue culture flasks. At each 24 hour time point, 3 flasks were removed and the viable cells counted using the trypan blue methods described in the Materials and Methods Section. The results plotted on the above graph gave an approximate doubling time calculated as 14.5 hours.

Figure 5.3: tTGase activity in the P8 cell line at different confluency



Cells were taken at approximately 50% and 90% confluency, homogenized and assayed for tTGase activity using the [^{14}C]-putrescine incorporation into N,N'-dimethylcasein method described in the Materials and Methods Chapter. Data shown represents the mean of 3 experiments ± S.E.M. This change is not statistically significant (p=0.077412).

5.2.4 Production of high expressing tTGase P8 clones using cell transfection

In order to further investigate the role of tTGase in the P8 osteosarcoma, cells were transfected with the pSG5TG (carrying the cDNA for human tTGase) and pSVneo plasmid vectors using the DOTAP transfection system as described in the Materials and Methods Section.

Clones were selected for by the addition of 400μg/ml G418 to the normal growth medium and initially screened by staining with the CUB7402 anti tTGase antibody. Examples of positive, mixed population and negative clones are shown in Figure 5.4. Once the most positive clones were selected, the clones were assayed using the [^{14}C]-putrescine incorporation into N,N'-dimethylcasein method of tTGase activity as described in the Materials and Methods Section. Results are shown in Figure 5.5.

It was decided to use clones TG3 and TG4 in further experiments as these were the highest expressing clones. TG1 cells, which express similar levels of tTGase to the wild type were used as a negative control, along with the wild type cells.

5.2.5 tTGase on the P8 osteosarcoma cell surface

For many years, tTGase was believed to be an intracellular enzyme. However, more recently, there has been much research on the externalisation of the enzyme (Jones *et al.*, 1997, Gaudry *et al.*, 1999b, Akimov *et al.*, 2000). The majority of this work has demonstrated the involvement of the enzymes crosslinking ability in the stabilisation of the ECM (Aeschlimann *et al.*, 1996, Jones *et al.*, 1997). However, there is growing evidence for tTGase also being a cell surface adhesion protein important in cell attachment and spreading (Gaudry *et al.*, 1999b, Verderio *et al.*, 2001, Balklava *et al.*, 2002). To investigate this characteristic of tTGase at the cell surface in the P8 osteosarcoma cell line, cells were incubated in suspension with the fluorescently labelled primary amine substrate fluorescein cadaverine for 45

Figure 5.4: Examples of P8 clones being screened for tTGase by antibody staining

P8 clones produced were screened for high tTGase expression by antibody staining. Clones were grown in a 96-well plate for 36 hours. The culture medium was removed and the cells carefully washed twice in Dulbeccos PBS (1x). The cells were fixed with 4% NBF and stained with CUB7402 mAb as described in the Materials and Methods Section. (A) Shows a negative clone, (B) shows a clone of mixed population, and (C) shows a high expressing clone. The images were taken using an Olympus DP10 microscope digital camera attached to an Olympus CK2 light inverted microscope.

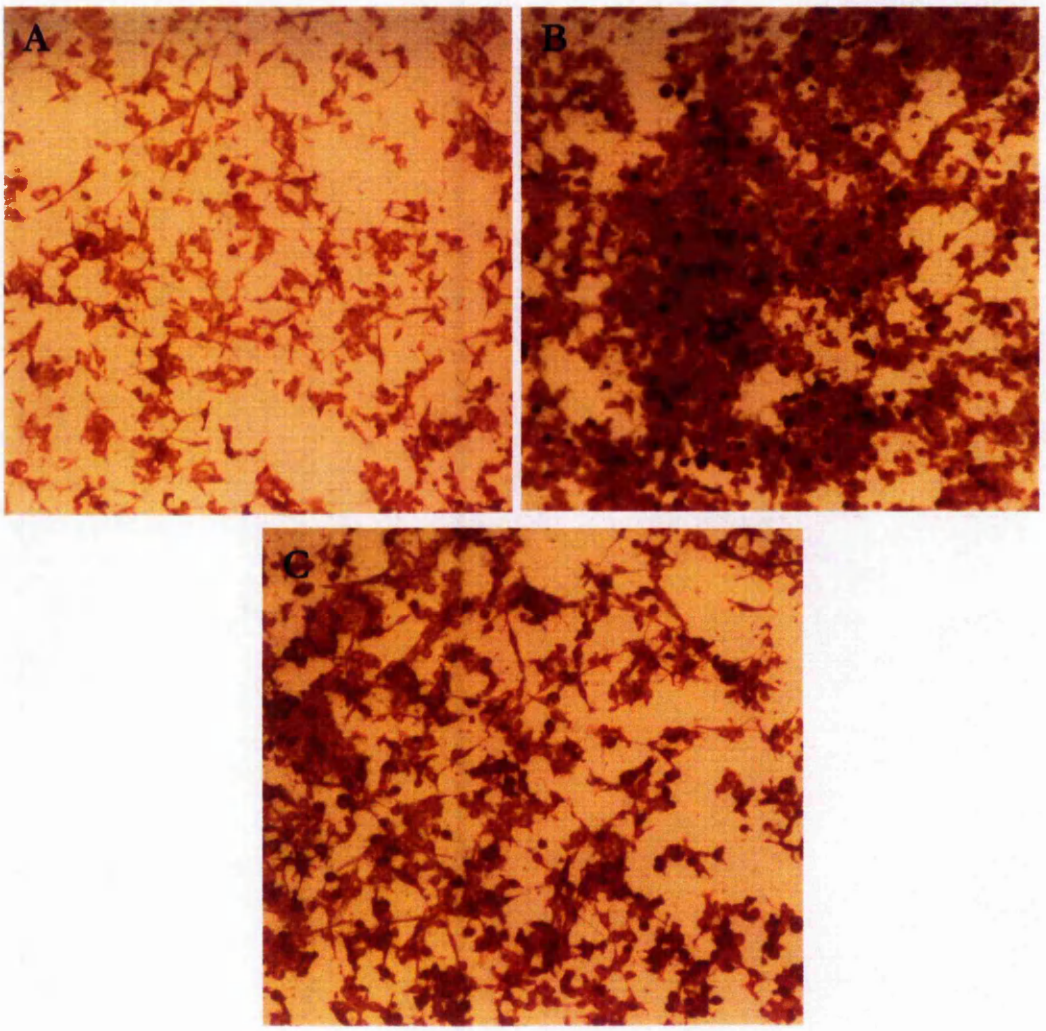
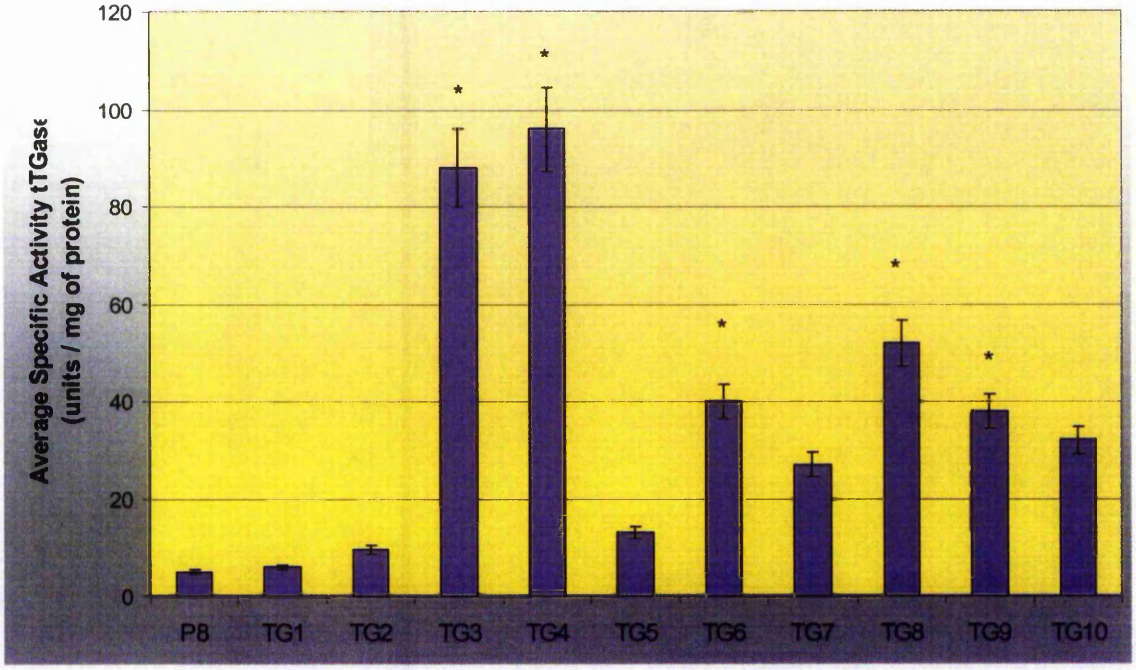

A
B
C

Figure 5.5: tTGase assay of potentially positive clones from above primary screening



*Potentially positive pSG5TG-transfected clones were assayed for tTGase activity by the [^{14}C]-putrescine incorporation into N,N'-dimethylcasein method as described in the Materials & Methods Chapter. Data expressed as mean of 3 samples ±S.E.M. *'s show statistically significant differences ($p \leq 0.05$) in tTGase activity to wild type P8 cells. Clones TG3 and TG4 were chosen for further use, and TG1 was chosen as a control clone with equivalent expressing tTGase to the wild type P8 cells.*

Figure 5.6: tTGase cell surface activity in the P8 osteosarcoma cell line

P8 cells were incubated in suspension with the 50μM fluorescein cadaverine for 45 mins. The cells were washed in PBS before being fixed in -20°C methanol and air dried onto a glass slide. After counterstaining with 100μg/ml propidium iodide and mounting the slide in Vecta-Sheild, the slides were viewed under a Leica Confocal Microscope. The sections above were set to 2μm steps. White arrow indicates cell dying from transglutaminase-associated cell death, yellow arrow indicates cell surface incorporation of fluorescein-cadaverine.

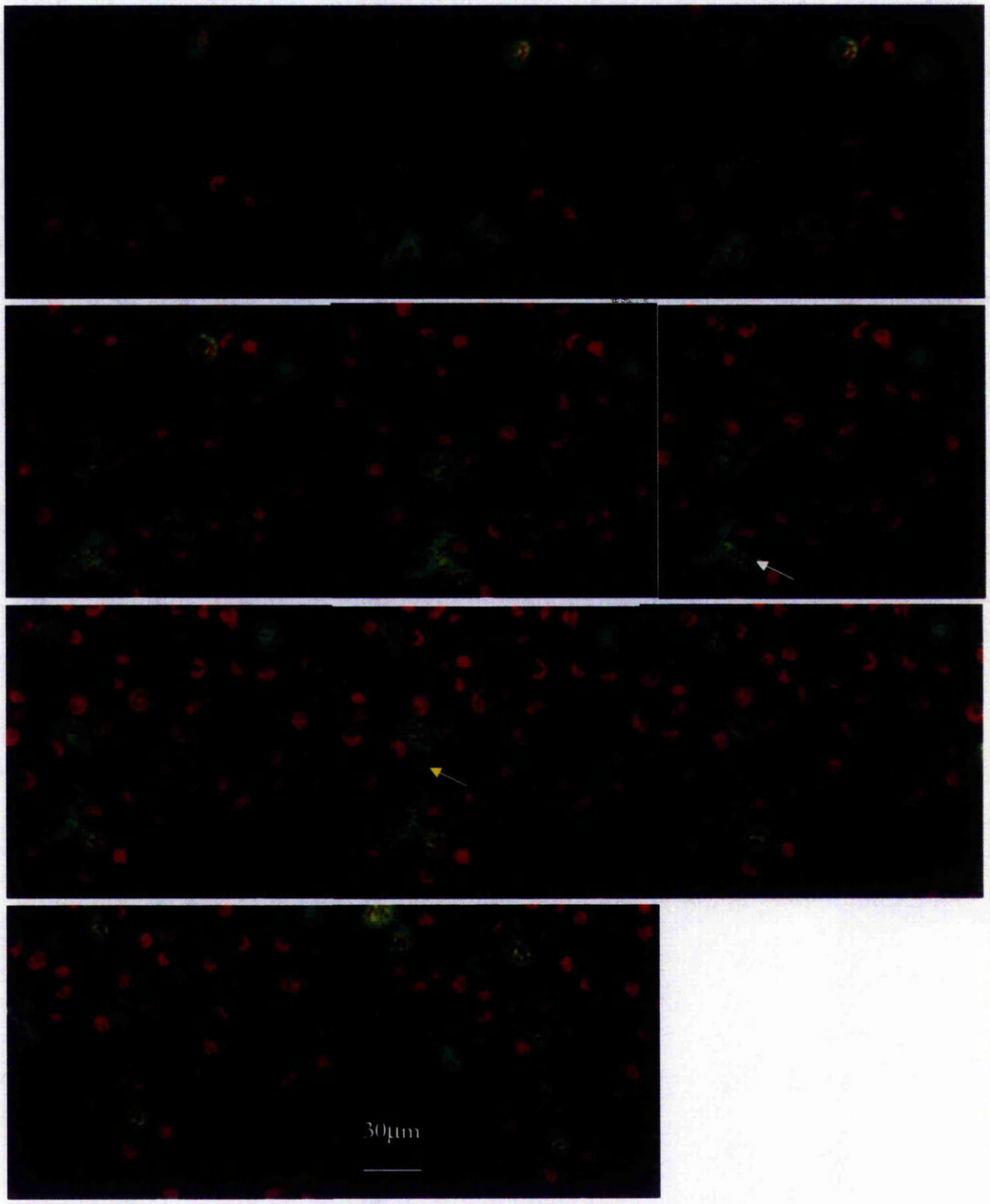

30μm

Figure 5.7: tTGase cell surface activity in the TG3 high tTGase clone

TG3 cells were incubated in suspension with the 50μM fluorescein cadaverine for 45 mins. The cells were washed in PBS before being fixed in -20°C methanol and air dried onto a glass slide. After counterstaining with 100μg/ml propidium iodide and mounting the slide in Vecta-Sheild, the slides were viewed under a Leica Confocal Microscope. The sections above were set to 2μm steps. White arrow indicates cell dying from transglutaminase-associated cell death, yellow arrow indicates cell surface incorporation of fluorescein-cadaverine.

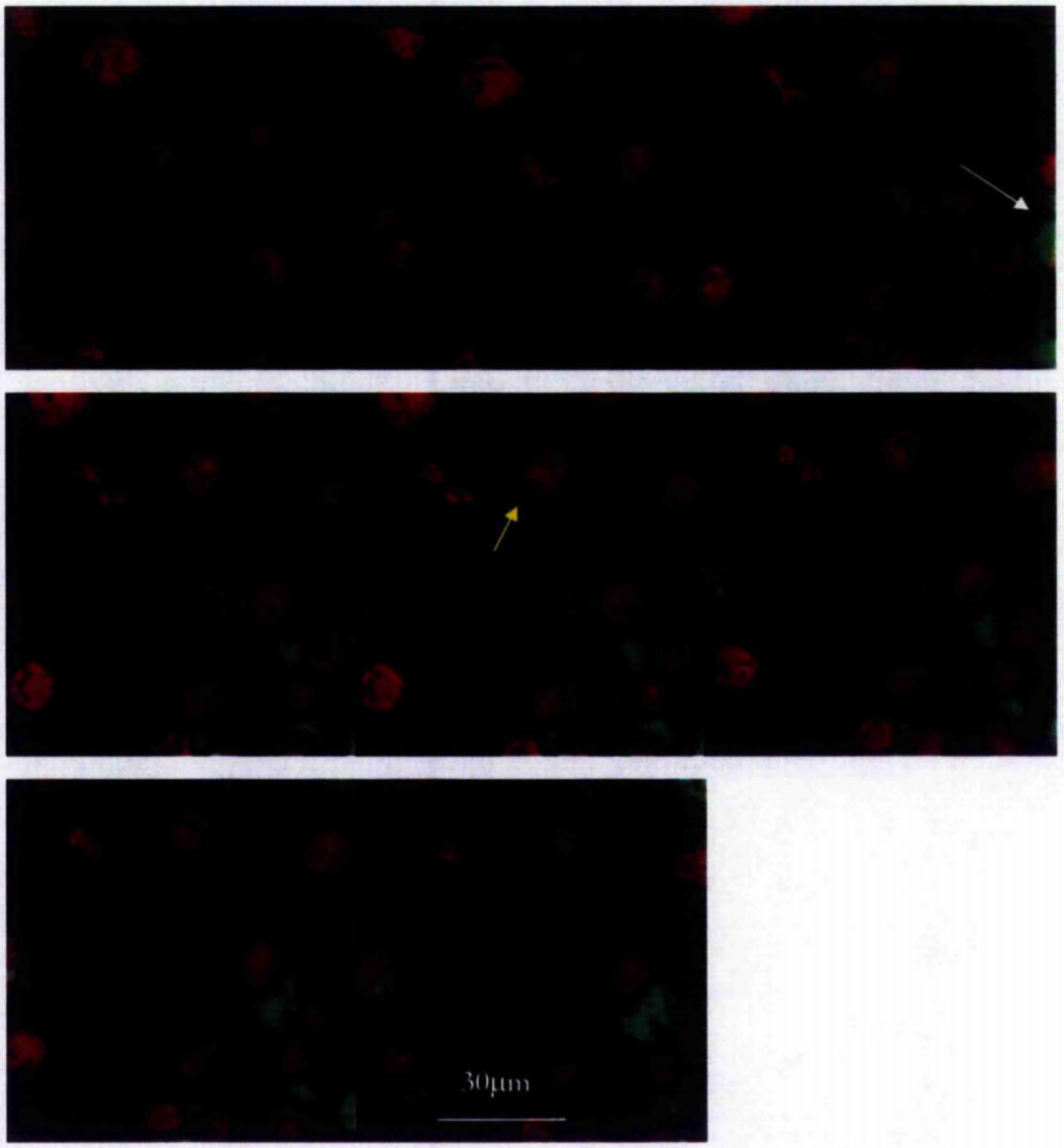

30μm

Figure 5.8: tTGase cell surface activity in the TG4 high tTGase clone

TG4 cells were incubated in suspension with the 50μM fluorescein cadaverine for 45 mins. The cells were washed in PBS before being fixed in -20°C methanol and air dried onto a glass slide. After counterstaining with 100μg/ml propidium iodide and mounting the slide in Vecta-Sheild, the slides were viewed under a Leica Confocal Microscope. The sections above were set to 2μm steps. White arrow indicates cell dying from transglutaminase-associated cell death, yellow arrow indicates cell surface incorporation of fluorescein-cadaverine.

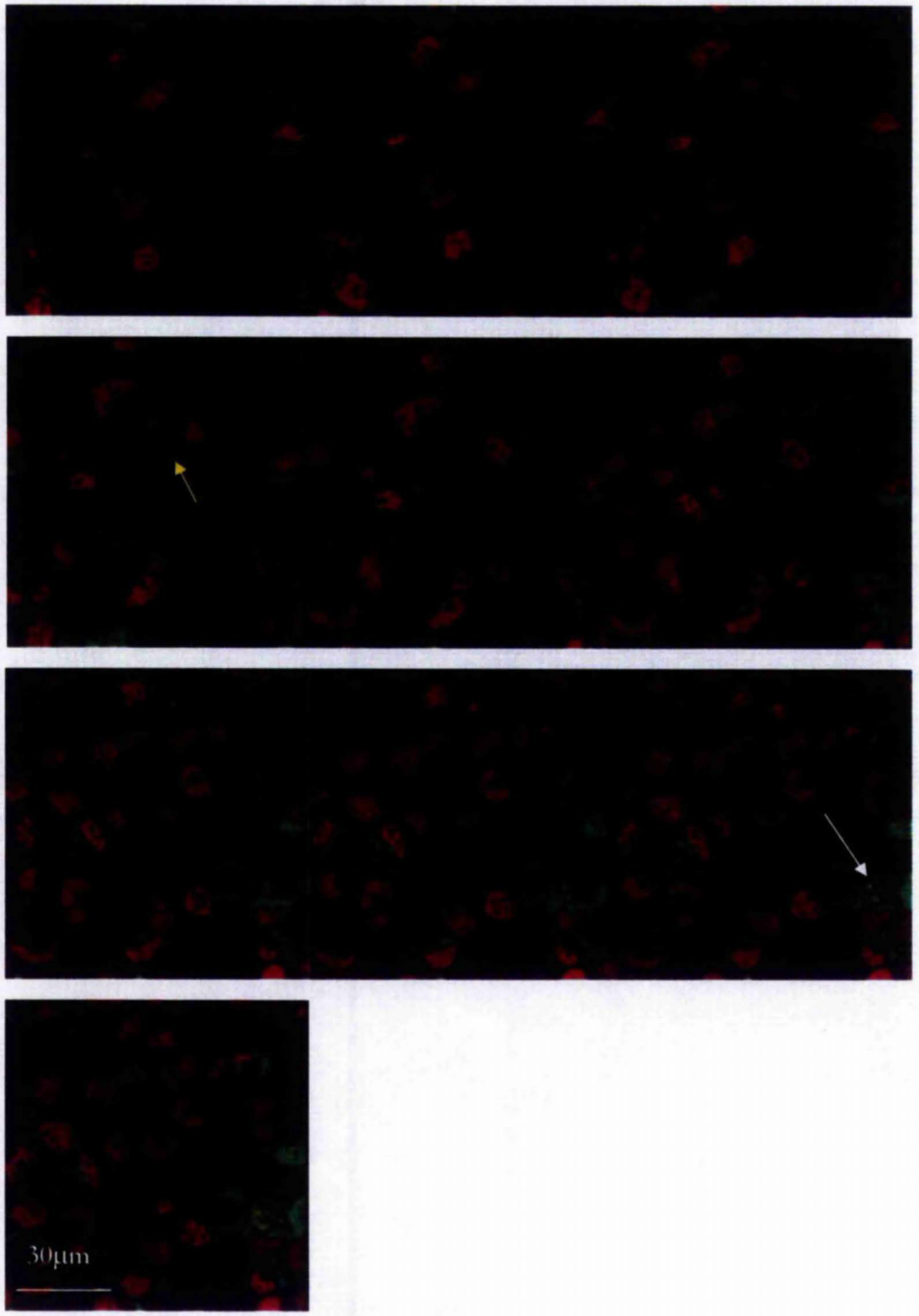
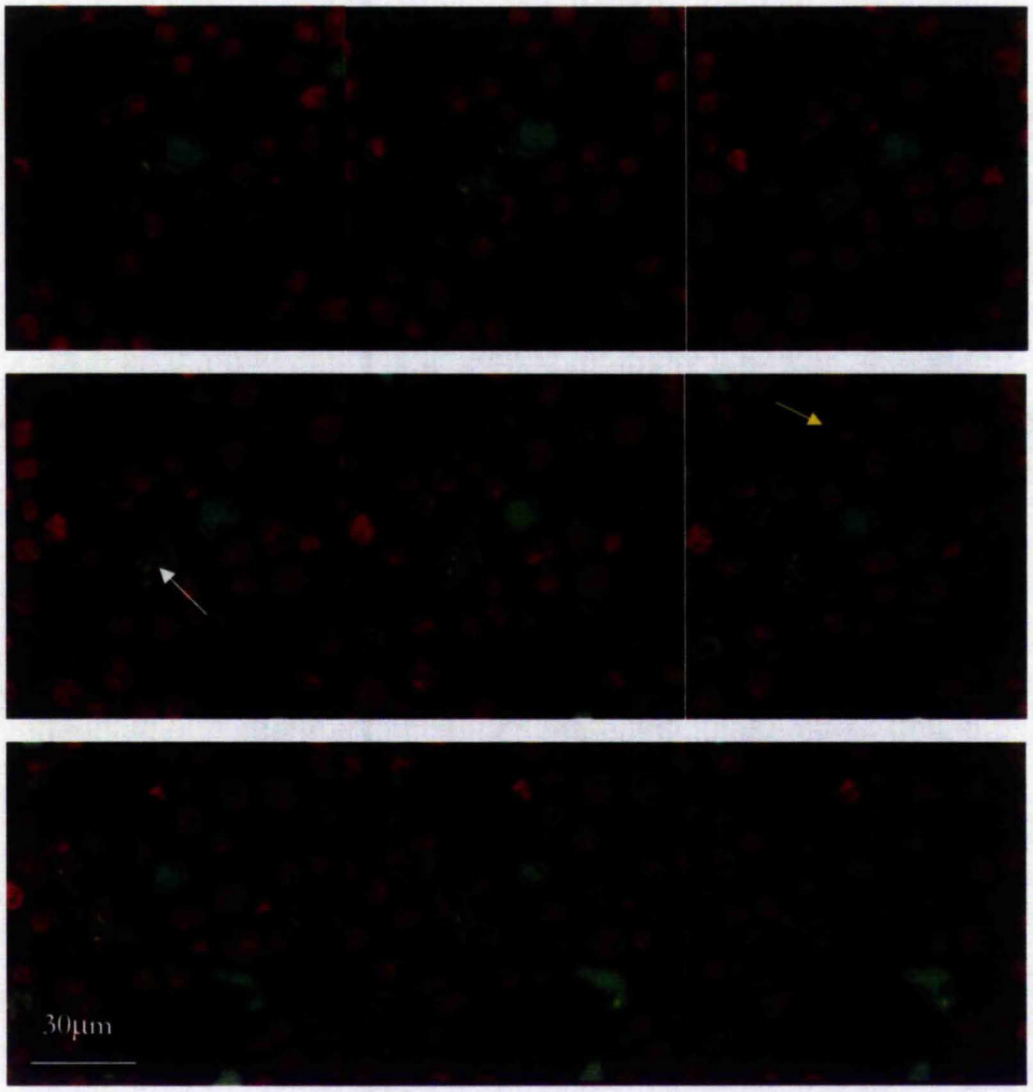

30µm

Figure 5.9: tTGase cell surface activity in the TG1 tTGase control clone

TG1 cells were incubated in suspension with the 50μM fluorescein cadaverine for 45 mins. The cells were washed in PBS before being fixed in -20oC methanol and air dried onto a glass slide. After counterstaining with 100μg/ml propidium iodide and mounting the slide in Vecta-Sheild, the slides were viewed under a Leica Confocal Microscope. The sections above were set to 2μm steps. White arrow indicates cell dying from transglutaminase-associated cell death, yellow arrow indicates cell surface incorporation of fluorescein-cadaverine.


30μm

mins. The cells were then washed in PBS before being fixed in -20°C methanol and air dried onto a glass slide. After counterstaining with 100μg/ml propidium iodide and mounting the slide in VectorShield, the slides were viewed under a Leica Confocal Microscope. The microscope was used to "slice" through the cells, giving a good picture of the cellular position of the activity of tTGase. The results show an increase in cell surface activity in the transfected clones when compared to the negative control (Figures 5.6-9), suggesting an increase in secretion of the enzyme in the high tTGase clones

It is interesting to note, that in all cases, although to a greater extent in the high tTGase clones, there are cells that have a high level of incorporation throughout the cell body, in the cytoplasm and nucleus. This is likely to be caused by rupturing of the cell membrane and the cell dying by transglutaminase-associated cell death as described earlier in the thesis.

5.2.6 tTGase crosslinking in the ECM of the P8 cell line

To further investigate the possible role for tTGase on the cell surface and in the ECM, the above experiment was repeated, but rather than the cells being kept in suspension, the cells were seeded into 8-well chamber slides and allowed to attach for 48 hours. Cells were washed in PBS before being fixed in -20°C methanol. The slides were counterstained using 100μg/ml propidium iodide, mounted using Vector-Shield and viewed under the Leica Confocal Microscope. The results show an increase in staining along the underside of the cell in all cases, with a greater level of staining in the high expressing tTGase clones when compared with the control. The wild type P8 cells also displayed a fairly high level of incorporation of fluorescein cadaverine underneath the cell (Figures 5.10-13). These results imply that tTGase crosslinking is important in the cell attachment process as it would appear to occur in the first three hours.

Figure 5.10: tTGase activity in the ECM of the P8 osteosarcoma cell line

P8 cells were seeded into a chamber slide in the presence of 50μM fluorescein cadaverine for 3 hours. Cells were washed in Dulbeccos PBS and fixed in -20°C methanol. Cells were counterstained with 100μg/ml propidium iodide and mounted in Vector-Shield before being viewed under a Leica Confocal Microscope. The sections above were set to 2μm steps.

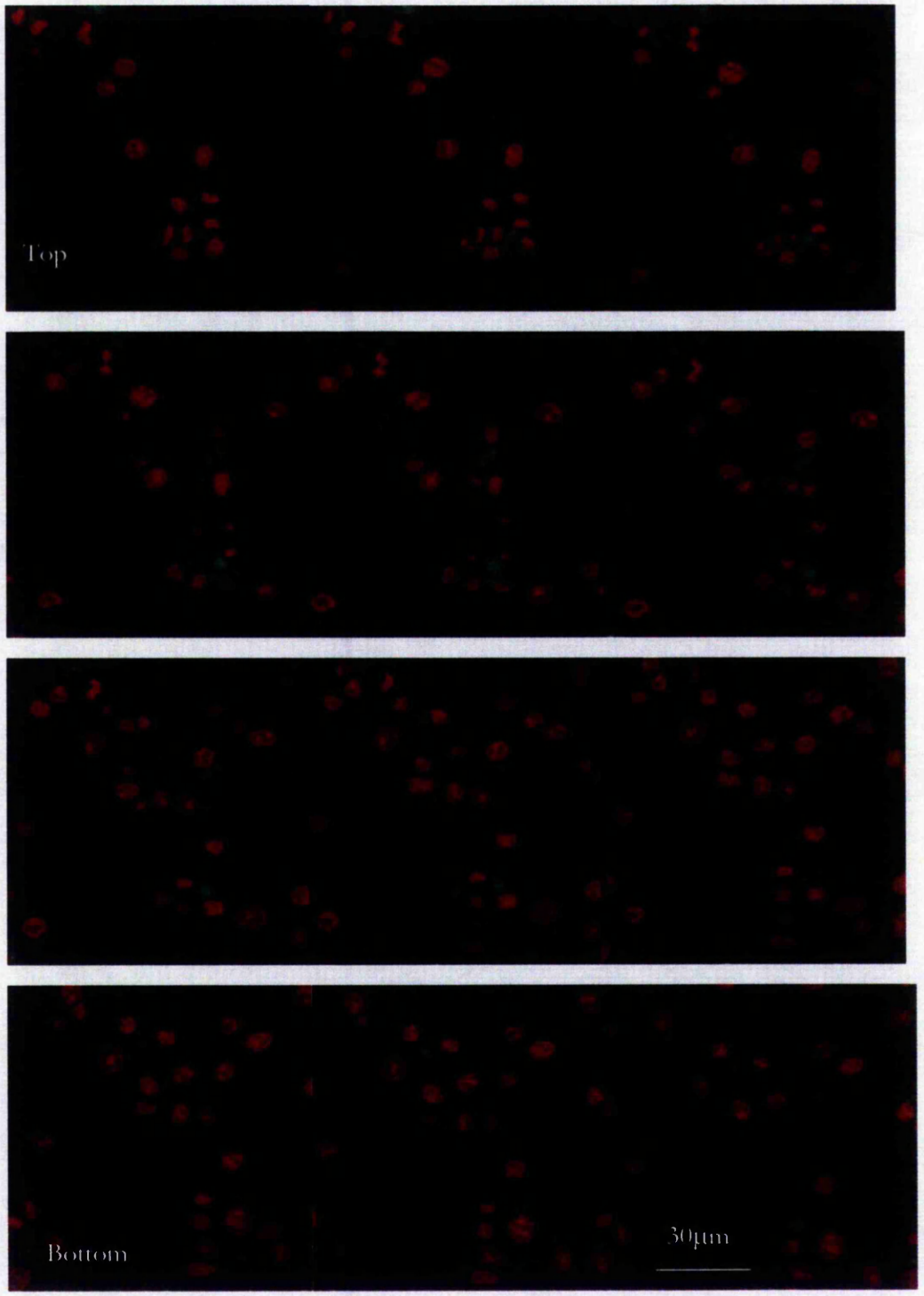

Top
Bottom
30μm

Figure 5.11: tTGase activity in the ECM of the TG3 high tTGase clone

TG3 cells were seeded into a chamber slide in the presence of 50μM fluorescein cadaverine for 3 hours. Cells were washed in Dulbeccos PBS and fixed in -20°C methanol. Cells were counterstained with 100μg/ml propidium iodide and mounted in Vector-Shield before being viewed under a Leica Confocal Microscope. The sections above were set to 2μm steps.

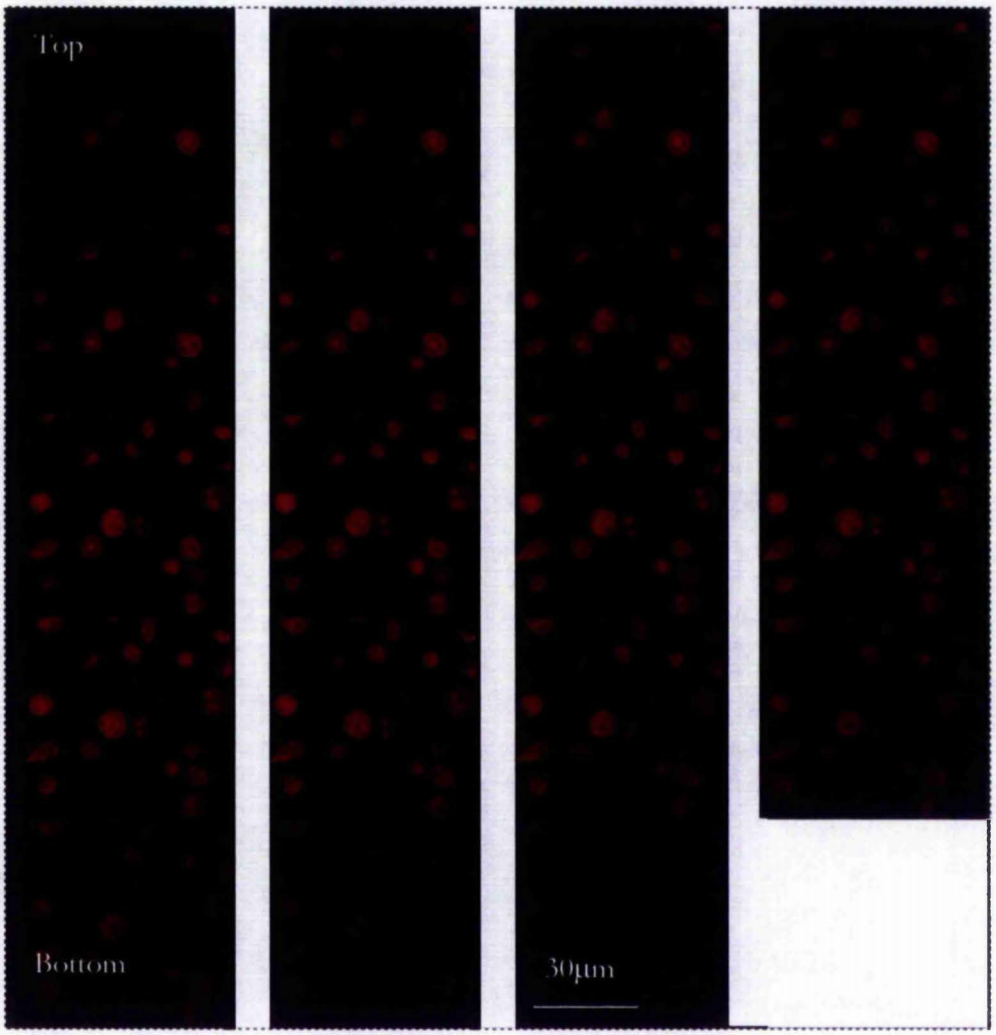

Top
Bottom
30μm

Figure 5.12: tTGase activity in the ECM of the TG4 high tTGase clone

TG4 cells were seeded into a chamber slide in the presence of 50μM fluorescein cadaverine for 3 hours. Cells were washed in Dulbeccos PBS and fixed in -20°C methanol. Cells were counterstained with 100μg/ml propidium iodide and mounted in Vector-Shield before being viewed under a Leica Confocal Microscope. The sections above were set to 2μm steps.

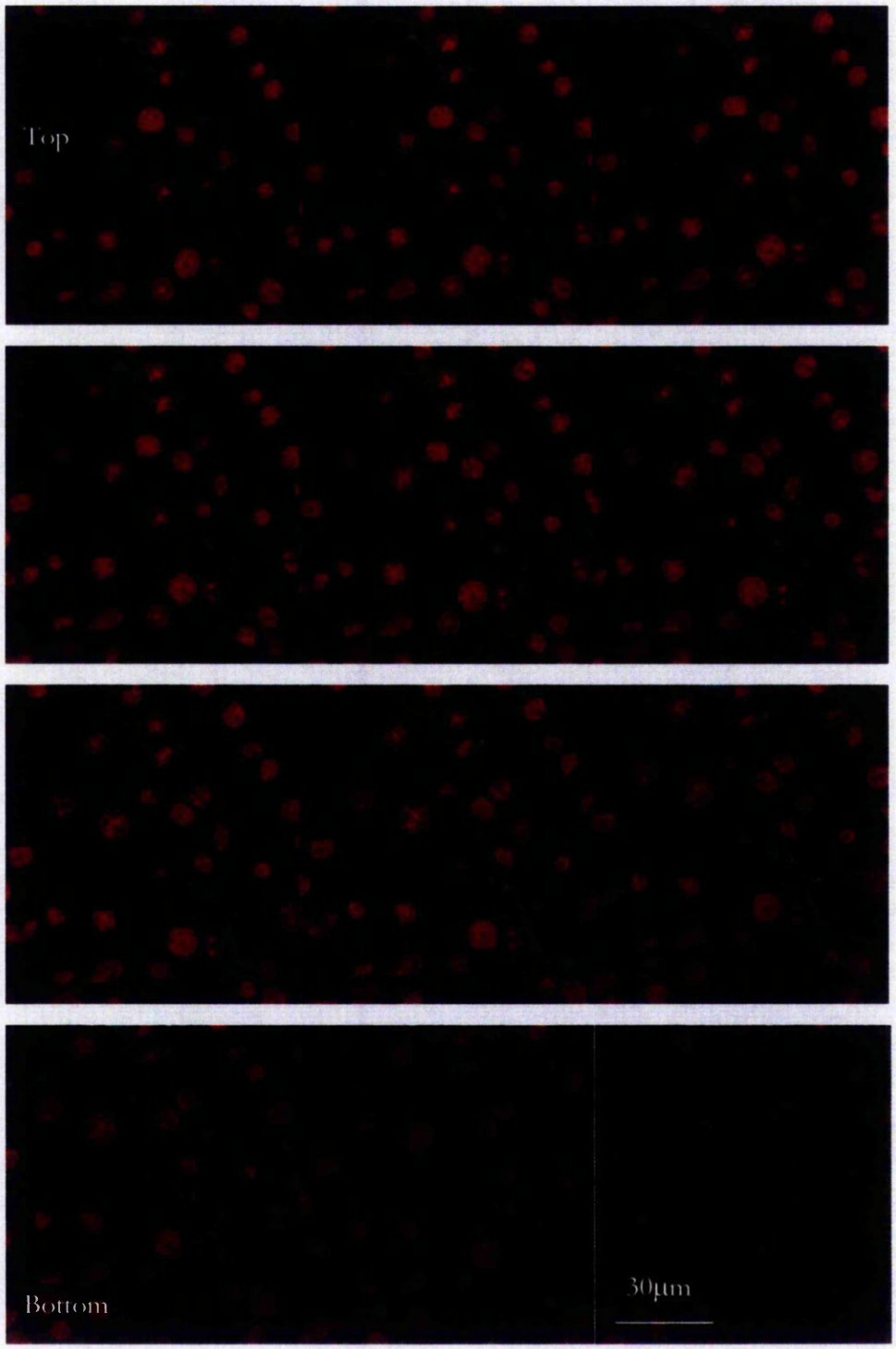
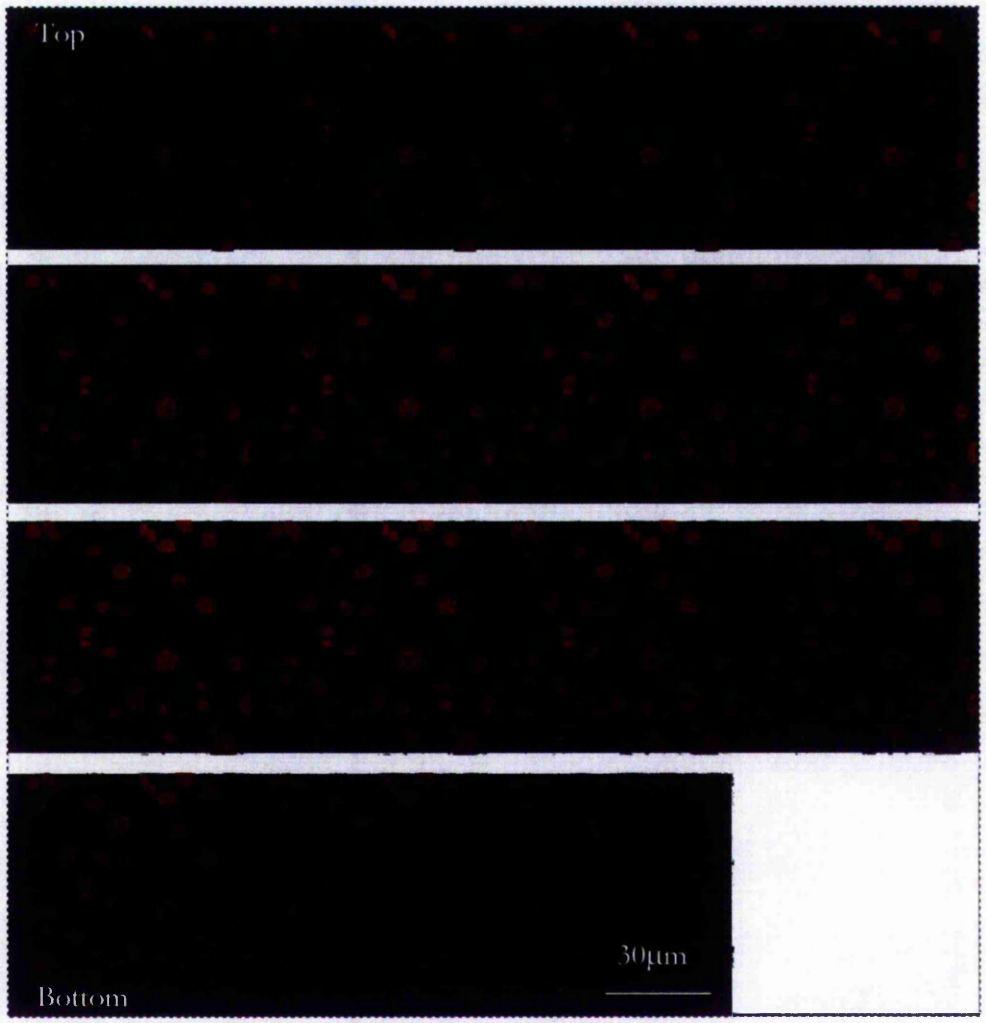

Top
Bottom
30μm

Figure 5.13: tTGase activity in the ECM of the TG1 tTGase control clone

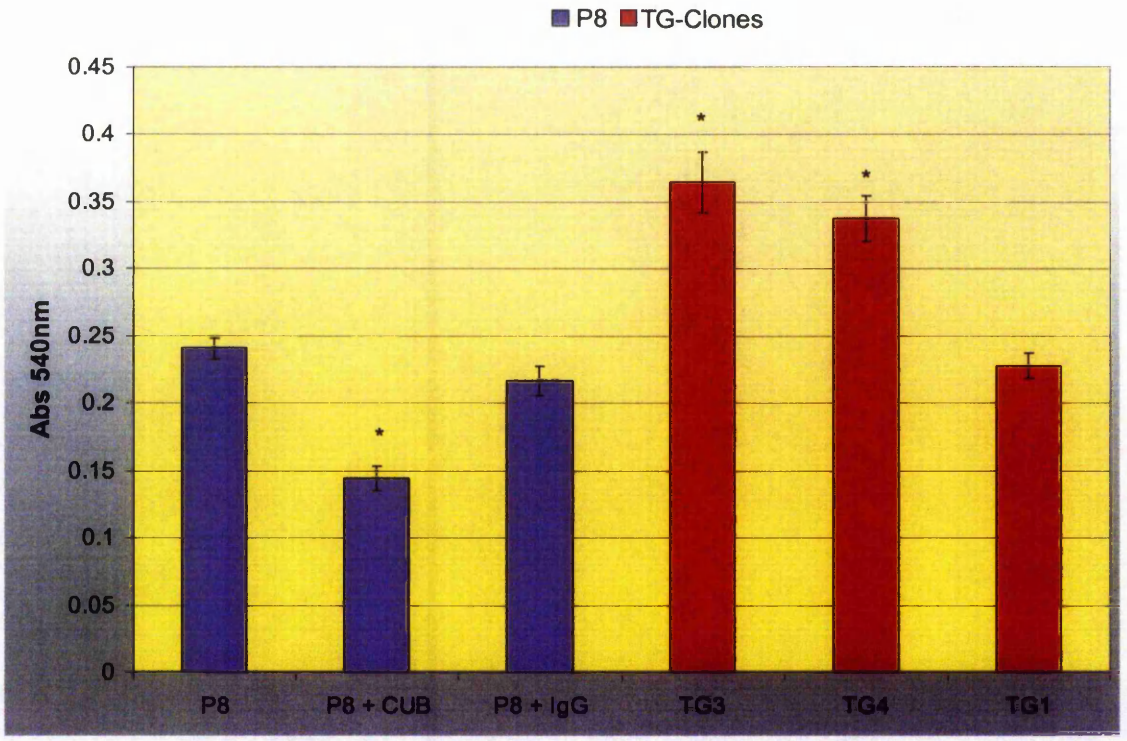
TG1 cells were seeded into a chamber slide in the presence of 50μM fluorescein cadaverine for 3 hours. Cells were washed in Dulbeccos PBS and fixed in -20oC methanol. Cells were counterstained with 100μg/ml propidium iodide and mounted in Vector-Shield before being viewed under a Leica Confocal Microscope. The sections above were set to 2μm steps.


Top
30μm
Bottom

5.2.7 The role of tTGase in cell attachment

tTGase has been shown to affect the ability of a cell to attach to both tissue culture plastic and fibronectin (Jones *et al.*, 1997, Verderio *et al.*, 1998). *In vivo*, this may be important in the colonisation of new tissue by tumour cells where they form secondary tumours (Kong and Korthuis, 1997, van_Groningen *et al.*, 1995). Cells were trypsinised, counted, resuspended in serum-free media and seeded into 96-well plates coated with fibronectin. The cells were incubated for 2 hours and the media removed. Cells were stained with crystal violet, solubilised and the absorbance at 540nm as described in the Materials and Methods Section. The results showed a statistically significant ($p<0.05$) reduction in cell attachment when the P8 cells were incubated with the CUB7402 anti-tTGase mAb. This reduction was not observed when P8 cells were incubated with non-specific IgG. This gives further evidence for both the presence of the enzyme on the cell surface and its involvement in cell attachment. Furthermore, the high expressing tTGase clones (TG3 and TG4) showed a statistically significant increase in cell attachment when compared with the TG1 control clone.

Figure 5.14: Cell attachment of P8 cells and high expressing tTGase clones on fibronectin



*Cells ($5x10^5$) were resuspended in 1ml of DMEM (900µl DMEM + 100µl of CUB or non-specific IgG as appropriate) and incubated for 1 hour at 37°C prior to plating out 100µl into wells of a 96-well plate coated with fibronectin. Cells were allowed to attach for 2 hours before the media was removed and the cells gently washed in PBS before 100µl of 0.5%(w/v) crystal violet was added to each well. The plate was incubated for 15 min at room temperature. Excess crystal violet was then removed by washing with PBS. Cells were solublised by the addition of 30% acetic acid and the absorbance at 540nm read on a plate reader. * show a statistically significant difference to the control (P8 or TG1), i.e. $p<0.05$ as determined by students t-test. The methodology is fully described Section 5.2.4 . Data represent the mean of 3 experiments ±S.E.M.*

5.2.8 Quantification of tTGase crosslinking at the cell surface

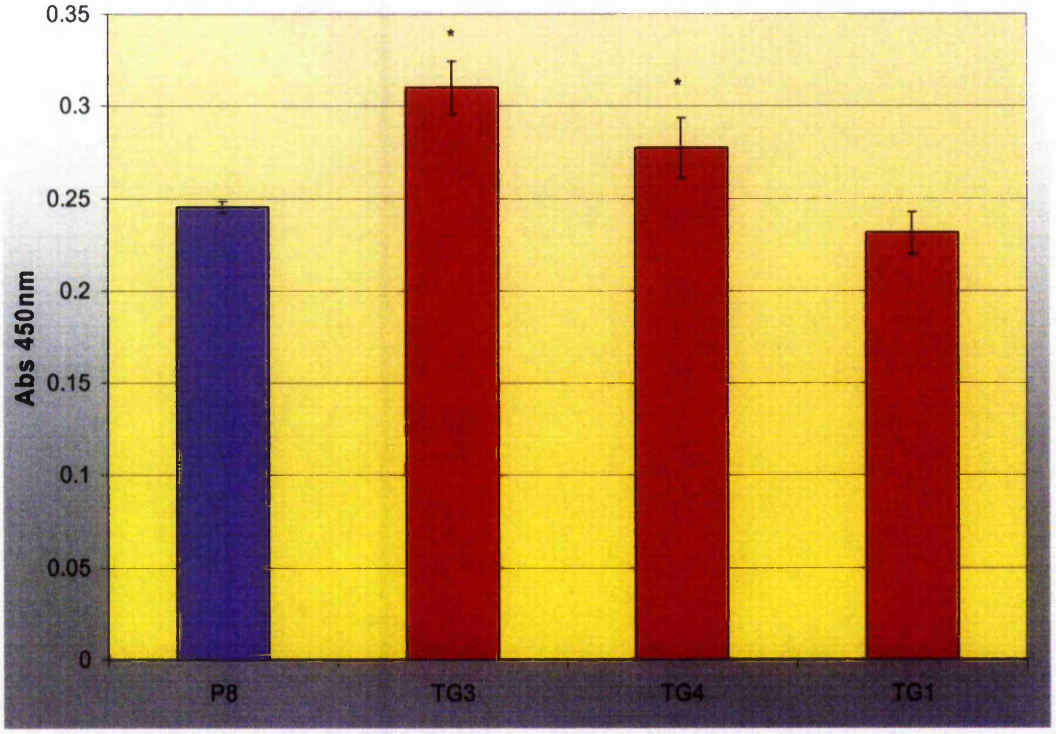
Having shown a qualitative increase of ECM crosslinking by the incorporation of fluorescein cadaverine, a more quantative method was used to validate this observation.

Cells were harvested and incubated in the presence of Biotin-X-Cadaverine and seeded into a 96-well plate pre-coated with fibronectin. Cells were left to settle for 2 hours. The cells were then lysed and extracted in the presence of EDTA, leaving the fibronectin and covalently incorporated Biotin-X-Cadaverine (Jones *et al.*, 1997). The results show a significant increase in incorporation of Biotin-X-Cadaverine in the high expressing clones tTGase clones TG3 and TG4 when compared with control clone TG1, which shows comparable incorporation to the wild type P8 cells (Figure 5.15), confirming the confocal data shown earlier.

5.2.9 tTGase bound into the cell surface/ECM

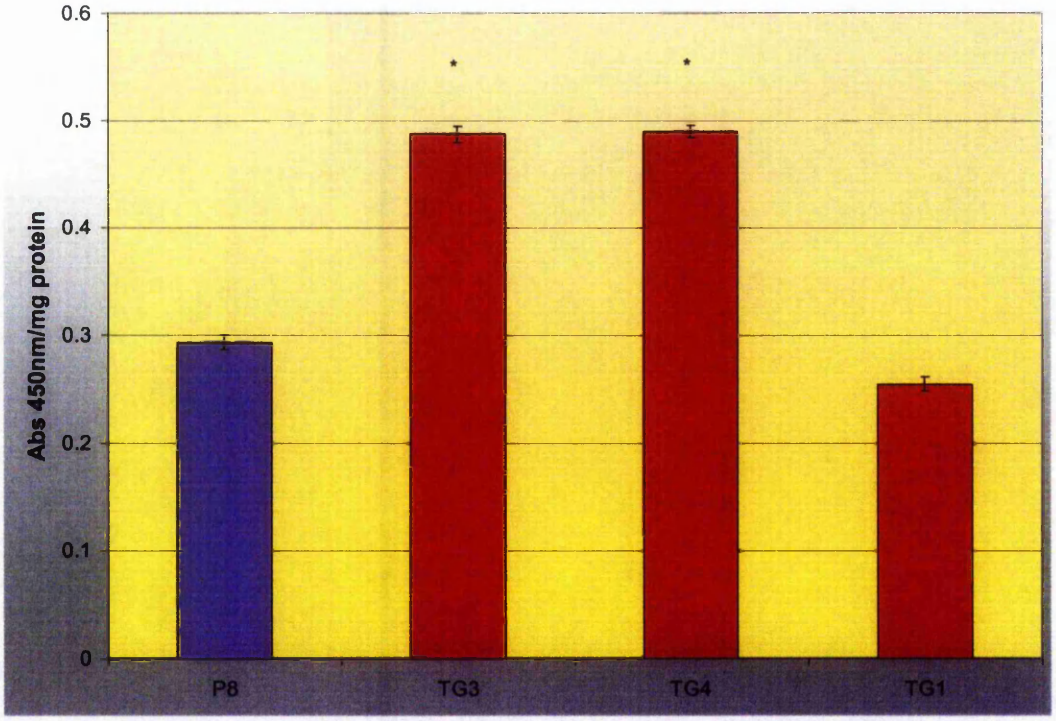
As we have shown an increase in crosslinking activity at the cell surface, the presence of tTGase in the extracellular environment was likely. In order to investigate this, a modified ELISA method (Verderio *et al.*, 1999) was used. This method initially involves the incubation of live cells with the CUB7402 anti-tTGase mAb. A standard ELISA protocol is then followed. The results confirmed that tTGase is a substrate for itself in the *in vitro* environment (Figure 5.16). This correlates with the immunohistochemical data shown in the previous chapter that tTGase incorporates itself into the ECM in the *in vivo* environment.

Figure 5.15: Incorporation of Biotin-X-Cadaverine into the ECM by tTGase



*Cells were harvested and resuspended in serum-free DMEM containing 0.132mM Biotin-X-Cadaverine. Cells were seeded into a 96-well plate coated with fibronectin and incubated for 2 hours at 37°C and 5% CO_2. Cells were washed carefully in PBS (pH7.4) before being solubilised with 0.1% deoxycholate in 2mM EDTA/PBS (pH7.4). The wells were developed by the addition of Extravidin peroxidase (dilution 1:5000) in 3% BSA in Tris-HCl (pH7.4), incubated at 37°C for 1 hour. After washing, the incorporation was revealed using 7.5%(w/v) TMB. This reaction was stopped by the addition of 50µl 2.5M H_2SO_4 to each well. The absorbance read at 450nm. Data represent the mean of 3 experiments ±S.E.M. * indicates results with statistically significant differences ($p<0.05$) to the TG1 control (which gives a similar result to the wild type P8 cells), as determined by students t-test detailed in the Materials and Methods Section.*

Figure 5.16: Measurement of cell surface & ECM bound tTGase by modified ELISA



*Cells (1.5 x 10^4/well) were seeded into a 96-well plate and grown overnight. The extracellular pool of tTGase in wild type and transfected P8 cells was measured by the addition of CUB7402 anti-tTGase mAb to live cell cultures. Cells were then fixed in 3.7% paraformaldehyde and the antigen-antibody complex was revealed by incubation with a secondary anti-mouse IgG-HRP mAb. The bound HRP activity was detected by the addition of TMB substrate and absorbance readings at 450nm taken as described in Section 5.2.7. Absorbance levels were normalised to 1mg of deoxycholate extracted protein. Data represent the mean of 3 experiments ±S.E.M. *'s mark results with statistically significant differences ($p<0.05$) to the TG1 control, as determined by students t-test.*

5.2.10 Reintroduction of P8 clones into AS rats

With the production of high expressing tTGase clones, it was decided to reinject cells back into AS rats to determine the effect, if any, of maintaining a high level of tTGase throughout tumour growth. In addition, since the formation of metastases appeared to correlate with a high level of tTGase, experimental metastases experiments involving the injection of P8 cells into the rat tail vein was also performed in order to monitor differences in colonisation of the lungs (preferred organ of metastasis) between the high expressing and control tTGase clones.

Cells were trypsinised and washed several times in serum-free media in order to remove any potentially immunogenic foetal calf serum from the cells. The cells were finally resuspended in Dulbeccos PBS and injected sub-cutaneously into the flank of 8 AS rats and the tail vein of another 8 AS rats for each cell type (P8, TG3, TG4 and TG1).

Tumour growth was expected to be observed within 2 months as the work on the solid tumour implants was completely over in this time. Tumours appeared on the flanks of 4 of the rats injected with the P8 cells. The tumours were allowed to grow to a diameter of 2cm (a size where secondary tumours had been previously easily detected) and then the rat killed by cardiac puncture and the lungs stained with Indian ink for identification of secondary metastatic tumours. In no cases were any metastases present. No other tumours appeared on the rats.

In the rats injected with cells into the tail vein showed no signs of tumour growth or ill-health 10 weeks after injection. Rats were sacrificed and a post-mortem carried out. None of the rats exhibited any form of tumour growth in their lungs or liver.

Both experiments were repeated using a wider bore needle for injections in case cells had been ruptured during the injection. Again, limited primary

tumour growth (just under 50% of animals) occurred only in the wild type P8. This time, to improve the chances of detecting secondary tumours, primary tumours were resected at 1cm diameter (the cut-off point for resection as governed by the home office licence), but after another 4 weeks, apparently healthy rats were culled and the post mortem revealed no secondary tumour growth. Similarly, in the experimental metastases model, no tumours were detected in the lungs of any of the animals.

5.3 Discussion

Cells isolated from solid P8 rat osteosarcoma have been shown to exhibit a number of similar traits to the tumour, providing an *in vitro* tool for studying the development of the tumour. Data in this chapter demonstrates, in the rat osteosarcoma cell line, tTGase is expressed on the cell surface and maintains this activity in the ECM in both the wild type and to a greater extent in the high expressing tTGase clones. This cell surface expression and crosslinking activity is more apparent when the cells are attached, with the tTGase competitive primary amine substrate fluorescein-cadaverine being detected along the underside of the cell. This is consistent with findings from other cell lines (Jones *et al.*, 1997, Gaudry *et al.*, 1999a, Belkin *et al.*, 2001). The increased presence of the enzyme itself, and the increased activity at the cell surface, would appear to increase the capability of the cells to attach to fibronectin. This could be due to increased fibronectin crosslinking, which may make it a better substrate for attachment (Barsigian *et al.*, 1991), or as recent research suggests, as a co-receptor for integrin binding to fibronectin (Akimov *et al.*, 2000). However, the results would suggest the tTGase-mediated crosslinking does play an important role in cell attachment as increased crosslinking activity could be correlated to increased cell attachment.

Unfortunately, the high expressing tTGase clones did not grow into either primary or experimentally induced "secondary" tumours. This could be for a number of reasons. Firstly, the tTGase cDNA used was human in origin, not rat. This may have provoked an adverse immune reaction causing the rats to reject the cells and stop the tumour from forming. Secondly, tTGase containing cells are actively selected against during tumour growth. This would correlate with previous findings by other researchers (Johnson *et al.*, 1994). Thirdly, tumours are made up of a heterogeneous population of cell types (Marsh *et al.*, 1993). It is possible that non-metastasising clones outgrew

the metastasising clones in the initial stages of cell culture, eventually becoming a "pure" culture. This would possibly have been made worse when selecting out the positive and negative clones from transfection, or that one cell type survived better in liquid nitrogen storage better than the other(s). It is also possible that increased tTGase may increase survival by helping these cells to attach and spread better (Griffin, *personal communication*), giving weight to the theory of clonal selection in the wild type P8 cells. Lastly, the cells may have mutated in culture or storage. I think this is probably the most likely explanation as cell types have been shown to change characteristics when in cell culture after several passages (Ciccarone *et al.*, 1989, Ross *et al.*, 1983, Beidler *et al.*, 1973).

However, the results from the above experiments showed that increased expression of tTGase leads to increased adhesion and ECM crosslinking. This is consistent with the solid tumour studies described in the previous chapter. This set of experiments would add weight to the theory that tTGase needs to be reduced in order for the tumour to progress to a metastatic state.

Chapter 6

GENERAL DISCUSSION

The role of tTGase in normal and abnormal cell function has been the focus of much research and the source of great controversy. The heterogeneous distribution of the enzyme together with its multiple catalytic activities has resulted in observations suggesting its involvement in many aspects of cell function. As a consequence, it has intrigued and frustrated researchers since the first identification of the enzyme in liver, over forty years ago (Clarke et al., 1957).

In recent years, tTGase has become increasingly important in the clinical arena. Currently, there is much interest in the role of tTGase in autoimmunity (Heitmeier et al., 1997, Lampasona et al., 1999, Piacentini and Colizzi, 1999) and neurodegenerative diseases (Lesort et al., 2000, Citron et al., 2002, Cooper et al., 2002, Piacentini et al., 2002), but one of the first pathological conditions that tTGase was proposed to play a role in was that of tumour growth (Yancey and Laki, 1972). Early work indicated a reduction in tTGase with cellular transformation (Birckbichler and Patterson, 1978) and tTGase levels are reduced in neoplastic tissue compared to normal tissue (Hand et al., 1987). However, later studies showed that this is not always the case (Takaku et al., 1995) and many tumour cell lines demonstrate high tTGase activity (Jones, 2001).

tTGase could potentially influence tumour progression and also tumour metastasis through a variety of mechanisms including:

- regulation of cell growth (Borge et al., 1996)

- ECM organisation (Menter et al., 1991)
- cell death (Knight et al., 1991)
- as a cell surface receptor (Belkin et al., 2001, Gaudry et al., 1999b)

Given the large amount of conflicting data on tTGase in this area, this Thesis sought to further clarify the role of tTGase in tumour growth and metastasis.

Using the previously partially-characterised transplantable P8 rat osteosarcoma, the tumour was first further characterised. Previous studies (Hand et al., 1987) had reported a high level of tTGase until a few days before metastasis was detected by staining the lungs with Indian ink. At this time, a sudden reduction in activity was noted. Hand *et al.* also found increased polyamine levels and covalently conjugated polyamines into tumour proteins. He postulated that protein crosslinking was blocked by polyamines thus regulating the production of the ε(γ-glutamyl) lysine crosslink.

In the work presented in this Thesis, the size of the tumour was used rather than the time from implantation as this appeared to give more reproducible results. It was found that tTGase activity and antigen drops as the tumour grows in size. Metastasis was determined after resection of the primary tumour, giving a far more accurate determination of time, or in this case, the size of the tumour and changes in tTGase activity. Secondary metastatic tumours were also investigated, which, in contrast to the primary tumour, showed an elevated tTGase activity and antigen. Given the increasing evidence for tTGase involvement in cell attachment and adhesion, the most likely explanation for this is that tTGase is reduced in order that it does not counteract the work of matrix metalloproteinases disrupting the ECM to facilitate tumour escape (Balklava et al., 2002). Reduction in tTGase has also been showed to facilitate the migration

of cells (Balklava et al., 2002). tTGase is high in the secondary metastatic tumours as it is needed to colonise the new tissue (Kong and Korthuis, 1997), possibly helping the cells attach to fibrillar fibronectin via its integrin co-receptor function (Belkin et al., 2001), or as an independent adhesion protein (Balklava et al., 2002). In postulating the involvement of tTGase in ECM organisation, several stains for ECM components revealed a fairly intact ECM and very limited angiogenesis into the tumour. This may however be due to the cartilage-like tissue that the P8 osteosarcoma forms. Cartilage ECM allows the passage of oxygen and nutrients (Buckwalter *et al.*, 1990). Cells isolated from the tumour showed incorporation of labelled primary amine substrates into the ECM, cell surface and basement membrane in this study gave further evidence of tTGase being expressed on the cell surface and helping the cell attach to and process the ECM. This has been shown in a number of other cell lines such as ECV304 (Jones et al., 1997), Swiss 3T3 fibroblasts (Verderio et al., 1998) and perhaps most interestingly in human osteoblast-like cells (Heath et al., 2001). Heath and co workers showed the importance of extracellular / cell surface tTGase in the interaction of these cells with ECM proteins, in particular fibronectin. Increased tTGase in human osteoblast-like cells have recently been shown to increase cell survival. Cell surface tTGase in these cells has been shown to help cells bind to fibronectin via an RGD-independent pathway, giving increased cell attachment and as a result increased cell survival (Verderio *et al., personal communication*).

The study of the P8 osteosarcoma has followed the regulation of tTGase throughout tumour growth and progression and as a consequence has given insight into the potential confusion which may arise while investigating the involvement of tTGase during tumour growth. Studies have suggested that tTGase is not a tumour-related marker (Takaku et al., 1995), based on the fact that tTGase levels differ between solid tumours and tumour cell lines and their normal counterparts. Work carried out in this Thesis would suggest this may be a

consequence of the timing of sampling. The change in expression of tTGase during tumour growth and metastasis was vast (a 10-fold increase in the secondary tumours compared to the hypothesised activity at the time of metastasis, although this could be 5000 times more activity in the secondary tumours compared to very large primary tumours) in the P8 osteosarcoma. If less time points were taken, very different results may have been observed. In fact, results in this thesis show a more gradual reduction in tTGase expression within the tumour when compared with the initial study (Hand et al., 1987). This is attributed to more frequent time-points measuring tTGase expression in this project.

Work carried out in this project also highlighted some of the problems faced with studying tissue-type transglutaminase. Experiments carried out using labelled competitive primary amine substrates are likely to be substrates for all transglutaminases. Reactions attributed to tTGase may be in fact catalysed by factor XIII or the more recently characterised transglutaminase X (Grenard et al., 2001a), as factor XIII has been detected in normal differentiating cartilage (Nurminskaya et al., 1998)

One of the primary goals of this work was to produce using cell transfection, high expressing tTGase clones from isolated P8 cells and investigate how these cells carrying high levels of tTGase formed and developed as tumours *in vivo*. Characterising these high expressing tTGase clones revealed they incorporate primary amine substrates into the cell surface, suggesting that at least some of the enzyme is externaliased. This higher level of incorporation is continued into the basement membrane of the cells, suggesting that the after transfection, the high expressing tTGase clones show an increased externalisation of the enzyme. This leads to an increase in cell attachment and matrix processing in these cell lines. This finding is in common with the findings of others using different cell lines

(Jones et al., 1997, Gaudry et al., 1999b, Belkin et al., 2001, Balklava et al., 2002, Heath et al., 2002).

The high expressing tTGase clones developed in this study, failed to grow into tumours, in keeping with the findings of (Johnson et al., 1994). The present data taken together with the work of Johnson *et al.*, strongly supports the hypothesis that high tTGase levels do not favour solid tumour growth. However, it should be noted that tumours were not formed in approximately half of the wild type cells, giving the possibility that the results observed could be a clonal effect, as clones expressing similar levels of tTGase to the wild type P8 also failed to form tumours.

The use of DNA arrays may also have elucidated a number of interesting result from the P8 cell and solid tumour work. The timing of the regulation of expression of matrix metalloproteinases in relation to tTGase expression *in vivo* would have given insight into the relationship between the antagonistic effects of those enzymes. Recent work has suggested that cell surface tTGase may be regulated by membrane-type matrix metalloproteinases (Belkin et al., 2001). The role of tTGase in tumour growth and progression is certainly a complicated one, which may be made clearer by investigating the regulation of genes thought to be working in tandem during tumour growth and progression. Unfortunately, problems with RNA isolation and time constraints prevented this work from being undertaken.

Another goal of this study was to assess the extent to which tTGase could be linked to the onset of tumour growth, via its postulated roles in apoptosis (Fesus et al., 1987). Knight *et al.* (Knight et al., 1991), showed a relationship between tTGase and metastatic potential. This investigation revealed that "Met" clones with higher tTGase activity were less likely to form secondary tumours. This

correlated with an increase in the number of apoptotic bodies isolated from these tumours. It was postulated that increased tTGase caused a higher level of apoptosis in these cells, thus reducing the apoptotic potential of these tumours. This study lead to an investigation into ways of inducing tTGase in these cells. Work by Johnson suggested that Met B hamster fibrosarcoma cells exposed to dexamethasone showed an increase in apoptosis as measured by the formation of apoptotic envelopes (Johnson, 1995).

The current study used more up to date and conclusive methods of apoptosis and revealed that this was in fact not the case. Data presented in this Thesis shows a good correlation of dexamethasone induction of tTGase with and increase in SDS-insoluble bodies, but no increase in classical apoptosis as determined by DNA fragmentation, induction of Capase-3 and ATP:ADP ratios. Cells transfected with the potent apoptosis inhibitor, bcl-2, also showed the formation of the SDS-insoluble shells. Cells treated with dexamethasone and apoptosis inducing compounds seemed to show a reduction in classical apoptosis when compared with cells not treated with dexamethasone. This implies that the chain of events leading to the new form of cell death observed, occurs more quickly than in the very structured and regimented apoptotic pathway. Given that dexamethasone treated cells appeared to be dying in a manner not consistent to either apoptosis or necrosis, it was concluded that the cells were dying by a new, uncharacterised form of cell death, termed transglutaminase-mediated cell death (Johnson et al., 1998).

Transglutaminase-mediated cell death occurs by loss of Ca^{2+} homeostasis, activating the tTGase inside the cell, which in turn crosslinks the abundant intracellular protein substrates, effectively fixing the cell *in situ*. This is comparable to the type of crosslinking observed in the terminally-differentiating keratinocyte (Bowness and Tarr, 1990).

Evidence of this type of cell death was also observed in the P8 cell lines and found to be occurring naturally in hamster liver and kidneys (tissues known to be high in tTGase). This type of cell death has now been discovered to be prevalent in kidney fibrosis leading to end stage renal scarring (Johnson et al., 1999), and may be important in other types of fibrosis.

Had more time been available, further studies on transglutaminase-mediated cell death would have involved a longer treatment of the tumours with dexamethasone and ionomycin and how this affects tumour weights and sizes. Also, any morphological changes within the tumour would have been an interesting addition to this study. The immunological potential of transglutaminase-mediated cell death should also be investigated, as this could have make a profound difference to the pathology of any disease states where transglutaminase-mediated cell death may occur.

Several factors also point to tTGase being a cell death component. The fact that the majority of the best crosslinking substrates are intracellular, yet the enzyme is not active under normal physiological conditions has perplexed researchers for years. The fact that the enzyme only becomes active when the membrane becomes "leaky" would be sensible. This may also go some way to explain current research into the role of tTGase in neurodegenerative disease. Researchers have suggested that alternate splicing may have removed the GTP regulation from the tTGase protein produced in patients suffering from Huntingdon's disease (Citron et al., 2002). This may make the enzyme far more susceptible to fluxes in intracellular Ca^{2+} concentrations, causing inappropriate crosslinking action. The enzyme has also been shown to crosslink the Tau protein in Alzheimer's disease, possibly by a similar mechanism (Miller and Johnson, 1995, Singer et al., 2002).

This type of cell death may also be important in the bodies natural defences against viruses. tTGase expression is induced by a number of cytokines involved in virus infection (Suto et al., 1993, Kuncio et al., 1998). As virus infection takes hold of the body, tTGase becomes upregluated in cells, but remains inactive due to the regulation of Ca^{2+} and GTP. As the virus replicates inside the cell, it packages itself into a glycoprotein coat, shown to be a substrate for tTGase (Beninati and Mukherjee, 1992, Mariniello et al., 1993). Viruses are released from cells by increases in intracellular Ca^{2+} concentrations.(Dayanithi *et al.*, 1995), this may activate tTGase causing the formation of a highly crosslinked mesh of intracellular proteins and virus. This would help stem the release of new viral infectious agents into the surrounding cells, thus helping prevent further viral infection. This is further supported by new research into tTGase and HIV infection, which shows an increase in cellular tTGase levels in cells infected by human immunodeficiency virus (HIV) (Amendola et al., 2001).

The recent production of tTGase-knockout mice has provided some surprises in the tTGase research community. These animals, as they age past 6 months, appear to be susceptible to glucose intolerance with mild hyperglycaemia due to a reduction in insulin secretion (Melino et al., 2001). With this exception, these animals appear to be mainly healthy, suggesting a non-essential role for tTGase in normal cellular function in the majority of cells. However, further analysis of these animals has revealed upregulation of other transglutaminase genes which may compensate for the loss of tTGase in various tissue (Jones, *personal communication*). These animals have also been shown to have impaired wound healing, possibly due to a reduction in cell migration (Mearns *et al.*, 2002).

As mentioned earlier, several of the roles for tTGase have been attributed as a consequence of there being tTGase substrates available, and tTGase involvement shown by the incorporation of labelled competitive primary amine substrates.

This has become more complicated in recent years by the discovery of three more transglutaminases (Grenard et al., 2001a). These uncharacterised enzymes could potentially be responsible for some of the reactions attributed to tTGase over the years.

The role of tTGase in the development of the malignant phenotype will not be fully understood until the role of tTGase in normal cellular function has been established. Tumours biology is a difficult area to study due to the complexity of the tumour mass being made up of a heterogeneous population of cell type, blood vessels and cells of the immune system along with fat cells and connective tissue can made up to 40% of the tumour mass. Also, researchers have a tendency to forget that different tumour types will behave in different ways, often depending on the origin of the tumour. The field is further complicated by the fact that primary tumours are not necessarily going to behave in the same way as the secondary metastatic tumours due to the clonal effect that occurs during the metastasis process.

On a more positive note, tTGase research into cancer has yielded some positive results. Intratumour injection of guinea pig liver tTGase appears to reduce the formation of neovasculature in certain tumour types (Griffin and Jones, 2001). However, when this was investigated using the P8 osteosarcoma, no effect was observed. This may have been due to the lack of angiogenesis in this tumour model, and the rapidity of growth that is observed in these tumours. The study also highlighted the difficulties in injecting solid bone / cartilage type tumours, as there is little dispersion of the agent throughout the tumour.

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