TRANSGLUTAMINASE ACTIVITY. TUMOUR GROWTH AND METASTASIS.

A Dissertation submitted to

The Council for National Academic Awards

by

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In Partial Fulfilment of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY.

AUGUST 1990

Faculty of Life Sciences

Nottingham Polytechnic

Nottingham

in collaboration with :-

The University of Sheffield

Department of Medical Microbiology and Virology, Tumour Biology Group

Sheffield.

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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.

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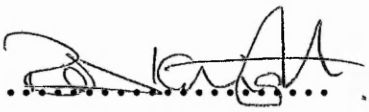
Signed  .

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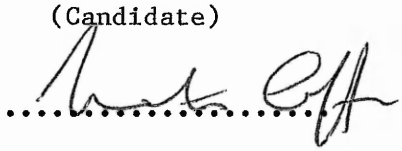
Signed  .

(Director of Studies)

To Philip, Angela and Brian.

ACKNOWLEDGEMENTS

Firstly I would like to express my thanks to Prof. M. Griffin for his support, encouragement and friendship over the last three years (and also for his confidence in me, without which this work could not have begun). I am also grateful to Dr. R. C. Rees and Dr. B. M. Elliott for their advice, assistance and friendship, and for giving me the opportunity to 'escape' from Nottingham Polytechnic occasionally!

I must thank Nottingham Polytechnic for provision of the facilities necessary for this project, and N.A.B. for financial support.

My thanks to all the workers in The Erasmus Darwin Building, Nottingham Polytechnic, for their help and camaraderie. Special thanks go to Jane, Janet, Jill, Louise, Mandy, Martin, Nigel, Paul and Roger for their technical advice and assistance, and also their tolerance. I would also like to thank everyone in the Tumour Biology Group at Sheffield University for their friendship and help, especially Andy, David, Janet and Lynda. Thanks are also due to the staff of Sheffield University Animal House for their help with the hamster studies.

Finally, I'd like to offer my commiserations to all those people who have had to work in the laboratory with me over the last three years : Ann, Dave, Jean, Jim, Jon, Julie, Karen, Kieran, Louise, Mark, Mash, Peter, Ruth and Tony.

TRANSGLUTAMINASE ACTIVITY, TUMOUR GROWTH AND METASTASIS.

C. Rosamund L. Knight

ABSTRACT

The aim of this study is to establish the importance of tissue transglutaminase activity in tumour progression and metastasis.

Results from metastatic variants, grown 'in vitro' and 'in vivo', indicated that cytosolic transglutaminase activity was inversely related to metastatic potential. Cytosolic transglutaminase activity was also seen to decrease during the growth of highly metastatic tumours. Particulate transglutaminase activity did not vary significantly between the variants, or during tumour growth.

A direct relationship between measured transglutaminase activity and the levels of $\epsilon(\gamma$-glutamyl)lysine (a product of transglutaminase activity) was found. This validated the use of an 'in vitro' activity assay for transglutaminase, and also suggested that $\epsilon(\gamma$-glutamyl)lysine crosslinks are the product of cytosolic transglutaminase activity.

Preliminary investigations into the cause of the reduction of cytosolic activity suggested an inactivation of the enzyme. Separation of antigenic transglutaminase protein by anion-exchange indicated the presence of an inactive form of transglutaminase that was clearly separable from the active particulate and cytosolic forms of the enzyme. Unlike neoplastic cells and tissues, the presence of an inactive form could not be demonstrated in control cells or normal liver. Measurement of antigen levels indicated an inverse relationship between the inactive protein and the active cytosolic enzyme, suggesting that the two forms are inter-related. From gel filtration, the molecular weight of the inactive form was found to be greater than both the particulate and cytosolic forms. Partial proteolysis of the inactive form led to its activation and to the appearance of a transglutaminase similar to the cytosolic transglutaminase.

Apoptotic envelopes, formed during programmed cell death, were found to contain greater than 80% of the cellular protein crosslink $\epsilon(\gamma$-glutamyl)lysine, indicating them to be highly crosslinked structures. A direct correlation was found between cytosolic transglutaminase activity and apoptosis, suggesting a role for this enzyme in programmed cell death.

A number of physical and immunological similarities between the particulate and cytosolic forms were observed. This suggests that the two forms may not be discrete enzymes, but may be modified products of the same gene.

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Biochem. Soc. Transact. (1989) 17, 714 -715.

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Immunological similarities between cytosolic and particulate tissue transglutaminase.
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FEBS Letts. (1990) 265, 93 - 96.

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Knight, C. R. L., Rees, R. C., Elliott, B. M. and Griffin, M.
19th Meeting of The Federation of European Biochemical Societies, 1989.

Is cytosolic tissue transglutaminase activity involved in the formation of apoptotic bodies in programmed cell death?
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LIST OF TABLES

LIST OF FIGURES

CONTENTS

1 INTRODUCTION:

1.1 An introduction to the Transglutaminases.

1.1.1 Definition.

Transglutaminases were first reported by Waelsch and coworkers in the 1950's (Sarkar et al, 1957; Clarke et al, 1957; Neidle et al, 1958; Clarke et al, 1959; Mycek et al, 1959). They identified, within guinea pig liver, a transamidating activity, dependent upon calcium, that could be measured by the incorporation of aliphatic amines into proteins. Much work has been published since that time, both on the original guinea pig liver tranglutaminase and on other transamidating enzymes. A large number of reviews have also been written on the subject of transglutaminase (Chung, 1972, 1975; Folk & Chung, 1973: Lorand & Stenberg, 1976; Folk & Finlayson, 1977; Folk, 1980; Williams-Ashman & Canellakis, 1980; Folk, 1983: Lorand & Conrad, 1984; Conrad, 1985).

For an enzyme to be described as a transglutaminase a number of criteria must be met. Enzyme activity must be calcium dependent with an active site thiol, thus enzyme activity can be inhibited by metal chelating agents (ie. EDTA and EGTA) and by thiol reactive compounds (ie. iodoacetamide). The catalysis reaction is an acyl transfer between peptide bound glutamine residues and primary amine groups. This results in the post-translational modification of proteins, by either the specific incorporation of amines, or, if the amine is the ϵ-amino group of protein bound lysine, the crosslinking of proteins via ϵ (γ-glutamyl)lysine bridges. From this it is apparent that the name 'transglutaminase' is inappropriate, since the glutamine which

participates in the reaction is peptide bound and not free. The enzyme commission therefore recommends the systematic name R-glutaminyl-peptide:amine-γ-glutamyl transferase (E.C. 2.3.2.13).

1.1.2 Classification.

Transglutaminases are found in a wide variety of tissues, both intra- and extra- cellularly (Folk, 1980; Conrad, 1985). Localisation and physical, chemical, immunochemical and catalytic properties are used to distinguish the transglutaminases (Chung, 1975).

Extracellular transglutaminases :-

Factor XIII found originally in plasma (Lorand et al, 1968), but now also in placenta (Bohn & Schwick, 1971), platelets (Schwartz et al, 1973), granulocytes, macrophages and monocytes (Berntorp et al, 1985). This enzyme is involved in the blood clotting cascade.

Prostate Transglutaminase found in rodent seminal plasma (Wing et al, 1974). Subsequent work has suggested that two forms of prostate transglutaminse exist (Chung, 1977; Wing, 1977; Lorand et al, 1979b; Tong, 1980; Williams-Ashman, et al, 1980). This enzyme is involved in the formation of the rodent copulatory plug.

Intracellular transglutaminases :-

Hair Follicle Transglutaminase found in mammalian hair follicles (Harding & Rogers, 1972; Chung & Folk, 1972). This enzyme is thought to play a role in the stabilisation of hair fibre (Peterson & Wuepper,1984).

Epidermal (Type 1) Transglutaminase found in the epidermis of many mammals (Buxman & Wuepper, 1974, 1975, 1976: Goldsmith et al,1974; Ogawa & Goldsmith, 1976; Peterson & Buxman, 1981). This enzyme is thought to play an important role in the formation of the cornified envelope during the terminal differentiation of keratinocytes (Rice & Green, 1979).

Tissue (Type 2) Transglutaminase found originally in the cytosol of many tissues (Sarkar et al, 1957; Chung, 1972; Lorand & Stenberg, 1976), but now thought to exist in a membrane associated form as well (Chang & Chung, 1986; Cocuzzi & Chung, 1986; Hand et al, 1988; Griffin et al, 1989). No specific function has as yet been established for this transglutaminase, though it has been postulated that it plays a role in calcium mediated cell membrane functions, and may be involved in the stabilisation of the cytoskeleton (Loewy & Matacic, 1981; Loewy et al, 1981; Bungay et al, 1984; Davies & Murtaugh, 1984; Conrad, 1985; Bungay et al, 1986).

The presence of calmodulin dependent transglutaminases in human platelets and red blood cells, as well as in chicken gizzard, has recently been indicated (Puszkin and Raghuraman, 1985; Puszkin and Billet, 1987). Recent work has suggested that the activity of erythrocyte transglutaminase is inhibited by the guanine nucleotides, GTP, GDP and GMP (Bergamini ei al, 1987). The nucleotide GTP reduces the affinity of the transglutaminase for calcium and alters the conformation of the enzyme (Bergamini, 1988). Lee et al, 1989, reported the hydrolysis of GTP by guinea pig liver transglutaminase, thereby demonstrating that the tissue enzyme has GTPase activity as well as transglutaminase activity. These systems therefore offer an alternative

regulatory mechanism for the incorporation of polyamines into proteins and the formation of the $\epsilon(\gamma$-glutamyl)lysine protein crosslink.

1.1.3 Transglutaminase 'in vivo' catalysed reactions.

Transglutaminases are capable of the catalysis of a wide variety of hydro- and amino- lytic reactions 'in vitro', however, only three transglutaminase catalysed reactions have so far been shown to occur 'in vivo'. These reactions are (Fig.1.1) :-

the incorporation of primary amines into peptides,

the crosslinking of proteins via the formation of $\epsilon(\gamma$-glutamyl) lysine,

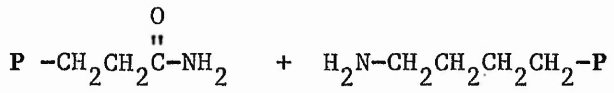
the crosslinking of proteins via the formation of N,N bis-γ-glutamyl polyamine derivatives.

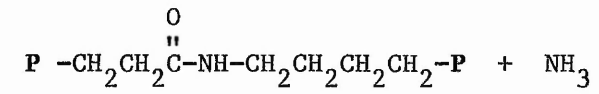
All of these reactions can be used to assess the activity of the enzyme, but it is the incorporation of primary amines into proteins that lends itself to 'in vitro' transglutaminase assays. The Ca^{2+} dependent incorporation of isotopically labelled amines, such as putrescine, histamine, spermine and cadaverine, into TCA precipitable proteins, such as casein or β-lactoglobulin, forms the basis of the original assay for transglutaminase (Lorand et al, 1972). Modification of the protein substrates by blocking the ϵ-lysine residues, by acetylation, succinylation, dimethylation or guanidylation, prevents self-crosslinking of the substrate and therefore gives improved results (Conrad, 1985). The incorporation of dansylcadaverine into protein substrates, giving an increase in the quantum yield of dansyl group emission, can also be used for assaying transglutaminase activity.

Fig. 1.1 : Transglutaminase catalysed reactions known to occur 'in vivo'.

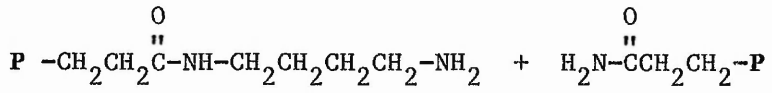
a) Incorporation of primary amines into proteins :

b) Formation of $\epsilon(\gamma$-glutamyl)lysine crosslinks :

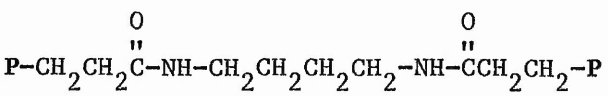




c) Formation of diamine crosslinks through the incorporation of polyamines (reaction product from (a) with putrescine as the primary amine) :



N-(γ-glutamyl) putrescine



N,N-bis-(γ-glutamyl) putrescine crosslink

1.1.4 The mechanism of catalysis.

Elucidation of the catalytic mechanism came from the isolation of a stable enzyme-substrate complex. The cysteine residue, which is essential for catalysis, was found to form a thioester intermediate with the peptide-bound γ-glutamyl residue. This could then undergo nucleophilic lysis by a primary amine or water. Kinetic analyses of the reactions catalysed by transglutaminases are consistent with a modified double-displacemant mechanism (Fig.1.2). A comprehensive review of the evidence in support of this is given by Folk (1983).

The presence of calcium ions is necessary for the activity of the enzyme (strontium and manganese can activate the enzyme to a lesser extent (Folk et al, 1967) and the sequencing of guinea pig liver transglutaminase (Ikura et al, 1987) and mouse macrophage transglutaminase (Stein et al, 1987), using cDNA technology, has indicated the presence of two glutamic acid rich domains that may be involved in the formation of calcium binding sites. By the same techniques, partial sequence homology has been shown between calcium binding proteins such as calmodulin and the transglutaminases Factor XIII (Putnam et al, 1987; Ichinose and Davie, 1988) and tissue transglutaminase (Stein et al, 1987). Furthermore, transglutaminase activation by calcium may bring about conformational changes in the protein structure which lead to exposure of the active site (Folk, 1983).

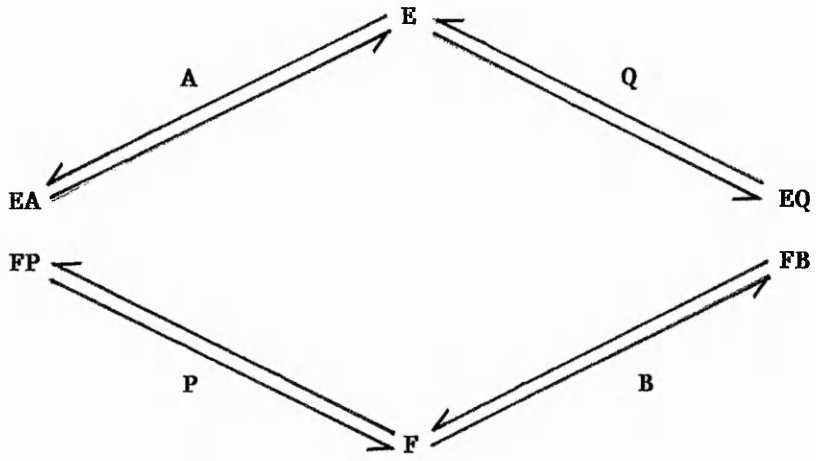
Sequencing of the guinea pig liver transglutaminase has indicated homology between the active site region of this enzyme and thiol proteases, such as cathepsin B, cathepsin H, papain and

actinidin (Ikura et al, 1987). Reactions of thiol proteases and transglutaminases proceed by common catalytic steps involving the formation of an acyl-enzyme intermediate at the active site cysteine residue prior to acyl-transfer. There is also homology between the active site of tissue transglutaminase from human endothelial cells, mouse macrophages (Gentile et al, 1990) and guinea pig liver (Ikura et al, 1987) and the active site of the plasma transglutaminase Factor XIII (Ichinose and Davie, 1988).

1.1.5 Energetic considerations.

The post-translational modifications of proteins catalysed by transglutaminases should be considered reversible since for every ϵ-amide bond broken there is a ϵ-amide bond created, thus very little change in free energy occurs. Campbell-Wilkes (1973) demonstrated the release of ^{14}C-histamine from β-lactoglobulin following its covalent incorporation by transglutaminase, indicating the reversibility of amine incorporation. However, due to the 'clotting' that occurs, it has been difficult to demonstrate the reversibility of protein crosslinking, and actual reversal has only been reported for the Factor XIIIa catalysed dimerization of fibrin with alpha$_2$-plasmin inhibitor (Ichinose and Aoki, 1982).

Fig. 1.2 : The mechanism of action of transglutaminase.



Key : reaction (b) from Fig.1.1 as an example

E = enzyme

F = acyl-enzyme intermediate

A = peptide bound γ-glutamine

P = ammonia

B = peptide bound ϵ-lysine

Q = $\epsilon(\gamma$-glutamyl)lysine

This is the reaction mechanism as it would apply to reaction (b) in Fig.1.1. Peptide bound γ-glutamine binds to transglutaminase to form an acyl-enzyme intermediate with the release of ammonia. This then undergoes nucleophilic attack by peptide bound ϵ-lysine to give the protein crosslink $\epsilon(\gamma$-glutamyl)lysine.

1.2 Biological roles for the crosslinking of proteins intracellularly.

In 1970, the presence of $\epsilon(\gamma$-glutamyl)lysine was discovered in digests of wool, hair and quills (Asqith, 1970; Harding and Rogers, 1970). Transglutaminase activity had previously been identified in wool by Rogers and Springell, 1959, and in 1972, Harding and Rogers demonstrated the existence of transglutaminase activity in hair follicles. Chung and Folk, 1972b, isolated two transglutaminases from hair follicle, one similar to tissue transglutaminase and the other one unique. It is widely believed that hair follicle transglutaminase plays a role in the stabilisation of the protein structure in the hair follicle by the formation of $\epsilon(\gamma$-glutamyl)lysine crosslinks (Rothnagel and Rogers, 1984; Peterson and Wuepper, 1984).

Transglutaminase activity has also been observed in the epidermis itself, and the 'epidermal' transglutaminase has been isolated from several sources (Goldsmith et al, 1974; Buxman and Wuepper, 1975; Ogawa and Goldsmith, 1976; Buxman et al, 1979; Negi et al, 1985). Buxman and Wuepper, 1975, showed that the enzyme was located in the granular region of the epidermis, where keratinisation was occurring and this suggested that it might be involved in the formation of the cornified envelope during terminal differentiation of keratinocytes. Indeed, it has now been shown that epidermal transglutaminase is involved in keratinocyte cornified envelope formation, again by the production of $\epsilon(\gamma$-glutamyl)lysine crosslinks (Green, 1977; Thacher and Rice, 1985; Schmidt et al, 1985; Jetten and Shirley, 1986; Michel and Demarchez, 1988).

Involvement of cell surface transglutaminase activity has

also been implied in the formation of the fertilization envelope of the sea urchin egg (Battaglia and Shapiro, 1988). Inhibition of transglutaminase activity leads in this system to disorganisation of the fertilization envelope and an increase in its permeability.

In ageing red blood cells (as well as in sickle cells and some other diseased red blood cells) the intra-cellular calcium concentration may rise from its normal level of 10^{-6}M, or below, to 2 x 10^{-4}M (Eaton et al, 1973; Palek, 1973) due to the failure of an outward directed calcium pump. Such a rise in the level of calcium would be sufficient to activate transglutaminase. Calcium loaded red blood cells, in the presence of the ionophore A23187 for more than a few minutes, suffer permanent shape change (White, 1976), loose their membrane deformability (Smith et al, 1981) and their membrane proteins undergo considerable structural changes (Lorand et al 1976, 1978, 1979; Siefring et al, 1978). High molecular weight polymers appear in the electrophoretic pattern of membranes from calcium treated cells and these polymers contain high levels of the protein crosslink $\epsilon(\gamma$-glutamyl)lysine, inhibitors of transglutaminase prevent this calcium effect (Lorand et al 1976, 1978, 1979; Siefring et al, 1978). Similarly, the production of non-disulphide linked polymeric material has been observed in calcium enriched rabbit lenses (a calcium dependent transglutaminase can be isolated from lenses) (Lorand et al, 1981; Azari et al, 1981; Conrad et al, 1983) and the formation of $\epsilon(\gamma$-glutamyl)lysine bridges andγ-glutamyl-putrescine has been reported in cataract proteins (Lorand et al, 1981; Kremzner et al, 1982 Valesco and Lorand, 1987). Further, β-crystallin chains from rabbit lens have been found to act as transglutaminase substrates, and antibodies raised against 'in vitro' formed polymers of rabbit β-crystallin chains

crossreact with a 50,000D protein polymer thought to participate in the formation of cataracts (Valesco and Lorand, 1987). Thus transglutaminase activity may be involved in the formation of lens cataracts.

The formation of paired helical filaments, in the brains of Alzheimer patients, by transglutaminase crosslinking has been reported (Selkoe et al, 1982a; Selkoe et al, 1982b) leading to the dysfunction and death of neuronal cells. Several workers have proposed that the crosslinking of proteins by $\epsilon(\gamma$-glutamyl)lysine bonds may be a general requirement for cells undergoing terminal differentiation in the process of cellular senescence (Lorand and Conrad, 1984; Birckbichler et al, 1988; Fesus and Thomazy, 1988) and recent reports have linked the activity of tissue transglutaminase with the process of programmed cell death (Fesus et al, 1987; 1988; Schmidt et al, 1988; Fesus et al, 1989). Fesus et al, (1987 and 1989), have shown that the apoptotic bodies formed during programmed cell death are insoluble in detergents such as SDS and contain $\epsilon(\gamma$-glutamyl)lysine and N,N-bis-γ-glutamyl-spermidine protein crosslinks, indicative of transglutaminase involvement. Hand et al, 1990, demonstrated the tissue transglutaminase mediated incorporation of methylamine into a heavy molecular weight 'polymer' by liver and hepatocellular carcinoma tissue slices and postulated that this 'polymer' might be apoptotic bodies formed by the action of the particulate form of tissue transglutaminase. Lower molecular weight proteins also acted as transglutaminase substrates in this system, notably Mr 39 000, 44 000 and > 200 000. Slife, 1990, identified proteins of Mr 230 000, 35 000 and 32 000 to be present in a heavy molecular weight polymer formed by transglutaminase. The 230 kD protein was identified as fibronectin and found to make up

approximately 25% of the polymer mass; Type IV collagen was also shown to be present in the polymer, making up approximately 20% of its mass; laminin, fibrinogen and type I collagen were were found not to be components of the polymer.

The action of transglutaminase has also been implicated in the crosslinking of fibrous proteins during pulmonary fibrosis (Richards and Curtis, 1984). Lung tissue transglutaminase is predominantly a particulate enzyme (Griffin et al, 1978; Cocuzzi and Chung, 1986) and it is thought to be involved in the formation of the extracellular matrix within lung tissue. In paraquat induced pulmonary fibrosis, the activity of lung transglutaminase was seen to increase, suggesting that the enzyme was involved in the irreversible crosslinking of proteins that occurs during fibrosis (Griffin et al, 1979). Richards and Curtis, 1984, postulated that the transglutaminase substrates present in the lung, such as fibrin and fibronectin, may well be crosslinked with collagen into insoluble complexes.

There are therefore distinct transglutaminases catalysing the formation of $\epsilon(\gamma$-glutamyl)lysine crosslinks in their respective tissues. This crosslinking appears to confer chemical resistance (either from external or internal sources) and mechanical strength to the resulting structures, and occurs both as a normal function of a tissue and as a result of some pathological conditions.

1.3 Proposed biological roles of tissue transglutaminase.

Type II transglutaminase, the 'tissue' transglutaminase is generally thought to be ubiquitous, its presence having been noted in a wide variety of animal cell types (section 1.1.2). By virtue of its widespread distribution, it can be hypothesised that the enzyme plays a role in one or more processes essential to the controlled normal functioning of the cell. This involvement could be via its function as a catalyst of protein crosslinking, or via its ability to incorporate polyamines into proteins. Both such mechanisms have the potential to alter the behaviour of the protein/s involved.

In the last few years, tissue transglutaminase has been implicated in a number of normal cellular processes. These include receptor mediated endocytosis, hormone secretion, cell activation and differentiation and the programmed deletion of cells. These are all calcium dependent processes and as such are potentially modulated by the activity of tissue transglutaminase. The activity of tissue transglutaminase, or rather the lack of such activity, has also been implicated in the 'abnormal' events of neoplasia and metaplasia, giving further support to the theory that tissue transglutaminase plays an important role in the normal functioning of cells.

1.3.1 Insulin secretion.

A rise in the concentration of free calcium ions within the cytosol plays a major role in the exocytic release of hormones such as insulin (Wollheim and Sharp, 1981). Work in this laboratory has shown

inhibition, by primary amines, of glucose mediated insulin release from the pancreatic beta-cell (Bungay et al, 1982; Bungay et al, 1984a), a finding supported by the work of Gomis et al, 1983. Bungay et al, 1984a, further demonstrated that the potency of primary amine substrates for transglutaminase was matched by their ability to inhibit insulin secretion although it was also demonstrated that primary amines may inhibit insulin secretion by interfering with targets other than transglutaminase (Bungay et al, 1984a; Sener et al, 1984; Bungay et al, 1986). Since proteins involved in secretion may be modified by phosphorylation, the relationship between protein phosphorylation and transglutaminase mediated protein crosslinking has recently undergone investigation (Owen et al, 1987). Transglutaminase inhibitors were found to significantly reduce the amount of ^{32}P associated with a high molecular weight, membrane-associated, phosphopolymer present in intact islets that had been glucose-stimulated. The formation of a similar polymer in calcium incubated islet homogenates is thought to be catalysed by transglutaminase (Bungay et al, 1986) thus it was suggested that phosphorylation and protein crosslinking reactions are closely linked in the islet beta -cell. Activation of the islet tissue transglutaminase could therefore result in the stabilisation, via crosslinking, of membrane or membrane-associated proteins. Were this to occur in predefined regions of the membrane, then transglutaminase could be involved in the directional movement of vesicles by the provision of anchorage points to the cytoskeletal network and/or the prevention of membrane intermixing during membrane recycling.

1.3.2 Receptor mediated endocytosis.

Various workers have implicated the activity of the tissue transglutaminase in receptor mediated endocytosis (RME) (Davies et al, 1980; Levitzki et al, 1980; Cheng et al, 1980; Haigler et al, 1980; Fitzgerald et al, 1980; Chuang, 1981, 1984; Pastan and Willingham, 1981; Tucciarone et al, 1981; Kuesch, 1981; Dickson et al, 1981; Davies and Murtaugh, 1984; Teshigawara et al, 1985; Hucho and Bandini, 1986; Seiving and Stenberg, 1988) by demonstrating inhibition of RME by the addition of competitive substrates of transglutaminase. Several groups (Van Leuven et al, 1980, Kaplan and Keogh, 1981 and Dickson et al, 1981) have suggested that reductions in RME, in the presence of methylamine or monodansylcadaverine, may be due to inhibition of receptor processing rather than a decrease in internalisation of receptor-ligand complexes.

Work from several laboratories has suggested that transglutaminase may be important in the function of macrophages since activated macrophages contain increased levels of tissue transglutaminase when compared to their non-activated counterparts, and this increase has been linked to an enhanced capacity for RME (Fesus et al, 1981; Schroff et al, 1981; Leu et al, 1982; Murtaugh et al, 1983). Inhibition of macrophage transglutaminase activity by cystamine blocks RME (Leu et al, 1982), and by methylamine or dansylcadaverine causes the inhibition of RME (Fesus et al, 1981; Leu et al, 1982). Fesus et al, 1982, suggested that transglutaminase might be involved in the processing of receptors in such a way as to promote ligand binding and phagocytosis.

Similarly, a survey of compounds capable of interfering with

the RME of alpha$_2$macroglobulin (α_2M) by fibroblasts identified several transglutaminase substrates and inhibitors (Maxfield et al, 1979; Davies et al, 1980; Levitzki et al, 1980). However, cells with low or undetectable levels of transglutaminase are still capable of endocytosising α_2M (Yarden et al, 1981; Davies, 1984) therefore indicating that tissue transglutaminase is not essential for its internalisation. This suggests that inhibition of transglutaminase may be affecting an accessory system such as the formation of cell surface aggregates which then affects RME.

1.3.3 Tissue transglutaminase and the cytoskeleton.

The cytoskeletal network found within all eukaryotic cells is involved in cellular motile events. Since this network of proteins is modulated by calcium it is therefore a potential target for transglutaminase catalysed post-translational modifications. Another modulator of cellular function is the extracellular matrix, which both modulates, and is modulated by, the cytoskeleton, and this too is a potential target for transglutaminase action.

Various cytoskeletal proteins, such as actin, α-actinin, cytokeratins, desmin, neurofilaments and the neurofilament peptides α- and β- tubulin and MAPs 1 and 2, tropomyosin, tubulin and microtubules, have been shown to act as transglutaminase substrates 'in vitro' (Derrick and Laki, 1966; Iwanij, 1977; Cohen et al, 1979, 1980; Gard and Lazarides, 1979; Selkoe et al, 1982; Conrad, 1985; Maccioni and Arechaga, 1986; Miller and Anderton, 1986; Zatloukal et al, 1989). Transglutaminase substrates also exist within the extracellular matrix, these include fibronectin, vitronectin and lipocortin I, which have all

been shown to act as substrates of transglutaminase 'in vitro' (Birckbichler and Patterson, 1978; Sane et al, 1988; Ando et al, 1990). The formation of $\epsilon(\gamma$-glutamyl)lysine protein crosslinks, mediated by transglutaminase, was first implicated in the modulation of the cytoskeleton by Loewy et al, 1981. They demonstrated that the level of cytoskeletal associated crosslink decreased in the presence of Mg^{2+}-ATP and was increased by the addition of Mg^{2+}-ATP plus Ca^{2+}. Indirect evidence to support the hypothesis of transglutaminase mediated cytoskeletal regulation has also been reported. The conversion of proinsulin to insulin requires the translocation of proinsulin and this conversion can be blocked by primary amines suggesting that transglutaminase may be involved in translocation (Alarcon, 1985). In support of this theory, Matrisian et al, 1987, reported that methylamine prevented the translocation of internalised epidermal growth factor within the cell. The blocking of microtubule formation by griseofulvin is associated with an increase in transglutaminase activity, although this could be due to an increase in intracellular calcium levels rather than an attempt to redress the balance within the cell (Denk et al, 1984). Maccioni and Seeds, 1985, suggested that transglutaminase might be involved in microtubule stabilisation, whilst Maccioni and Arechaga, 1986, implicated transglutaminase involvement in the cytoarchitectural changes occurring in the development of murine embryos for which cytoskeletal function is fundamental. Two groups of workers (Cariello and Nelson, 1985; Gagnon and Lamirande, 1986) have hypothesised transglutaminase involvement in regulating the interaction of flagellar sliding fragments by the cycling of $\epsilon(\gamma$-glutamyl)lysine bridges since a competitive substrate and a non-competitive inhibitor of transglutaminase both cause an increase in the forward swimmimg

speed of Arbacia spermatozoa. Calmodulin dependent transglutaminases have also been located, in chicken gizzard and human platelets and red blood cells, and these forms of the enzyme show high affinity for cytoskeletal proteins (Puszkin and Rughuraman, 1985; Puszkin and Billet, 1987).

Cytoskeletal protein interactions mediated by transglutaminase, may explain the implied involvement of this enzyme in exocytosis and endocytosis. Both systems require the translocation of vesicles either to, or to and from, the plasma membrane, and it has been proposed that microtubules and microfilaments are involved in this translocation process (Howell and Tyhurst, 1984).

Tyrrell et al, 1986, proposed that transglutaminase participates in cell adhesion by covalently crosslinking proteins at the sites of cell-cell contact. The adhesive extracellular glycoprotein, fibronectin (an 'in vitro' substrate of transglutaminase), has been identified within this matrix of crosslinked proteins (Tyrrell et al, 1988). Barsigian et al, 1987, and Fellin et al, 1988, have demonstrated the incorporation of fibrinogen and fibronectin into a high molecular weight complex at the cell surface through a mechanism consistent with transglutaminase mediation. Greenberg and co-workers, have identified tissue transglutaminase activity within endothelial cells capable of crosslinking and stabilizing fibrinogen (Greenberg et al, 1987), and have demonstrated the crosslinking of the adhesive extracellular glycoprotein vitronectin by guinea pig liver transglutaminase (Sane et al, 1988). They hypothesised that transglutaminase may provide another mechanism for the complexation of proteins within the extracellular matrix such as fibronectin, vitronectin, collagen and thrombospondin.

1.3.4 Cell activation and differentiation.

The activity of tissue transglutaminase has been proposed as a regulator of cell activation and differentiation in a variety of systems. These include lymphocyte and monocyte maturation (Schroff et al, 1981; Kannagi et al, 1982; Murtaugh et al, 1983), embryogenesis (Cariello et al, 1984; Maccioni and Arechaga, 1986; Uhl and Schindler, 1987), platelet activation (Barnes et al, 1985), red blood cell senescence (Lorand et al, 1976, 1978, 1979) chinese hamster ovary cell maturation (Milhaud et al, 1980; Scott et al, 1982), neuronal senescence (Selkoe et al, 1982a; Selkoe et al, 1982b) and cellular senescence (Lorand and Conrad, 1984; Birckbichler et al, 1988; Fesus and Thomazy, 1988). The former of these systems has been most widely studied. Stimulation of lymphocytes and monocytes has been shown to be associated with increased levels of transglutaminase activity (Fesus et al, 1981; Leu et al, 1982; Teshigawara et al, 1985); this increase has been shown to be due to rapid de novo synthesis of the enzyme (Murtaugh et al, 1983) caused by trans-retinoic acid stimulation of gene expression (Moore et al, 1984). This evidence has led to the supposition that transglutaminase is involved in the process of phagocytosis and further evidence to support this theory has been reported. Leu et al, 1982, demonstrated that inflammatory macrophages contain elevated levels of transglutaminase activity when compared to resident macrophages and primary amines have been shown to inhibit phagocytosis (Gunzler et al, 1982; Julian et al, 1983). It has also been shown that cell type and differentiation effect the level of activity of the enzyme (Schroff et al, 1981) such that differentiation

towards mature macrophage is accompanied by an increase in transglutaminase activity (Kannagi et al, 1982). The morphological differentiation of normal and leukaemic monocytes by stimulation with retinoic acid or its derivatives is also accompanied by a rapid increase in the synthesis of transglutaminase (Breitman et al, 1980; Davies et al, 1984; Moore et al, 1984; Maddox and Haddox, 1985; Mehta et al, 1986).

1.3.5 Tissue transglutaminase and the multistage model of carcinogenesis.

The possible association of tissue transglutaminase with such cellular functions as differentiation (section 1.3.4) cytoskeletal organisation (section 1.3.3) (and therefore potentially cell-cell contact) and cellular senescence (sections 1.2 and 1.3.4) raises the possibility of a potential role for transglutaminase in pathological events such as neoplasia. Laki and co-workers first proposed a connection between transglutaminase and tumour growth in 1966 when they described a fibrin stabilising activity (later known as tissue transglutaminase) in cell extracts from the mouse tumour YPC-1 (Laki et al, 1966). In 1972 they found that inhibitors of fibrin clot stabilisation increased the survival time of mice carrying the YPC-1 tumour (Yancey and Laki, 1972). Further, they demonstrated that tumours with low levels of transglutaminase activity were more likely to survive than tumours with high transglutaminase content (Fesus and Laki, 1976; Laki et al, 1977). Finally in 1977, they found a relationship between the tumour transglutaminase level and the transglutaminase content of the tissue to which that tumour

metastasises (Laki et al, 1977).

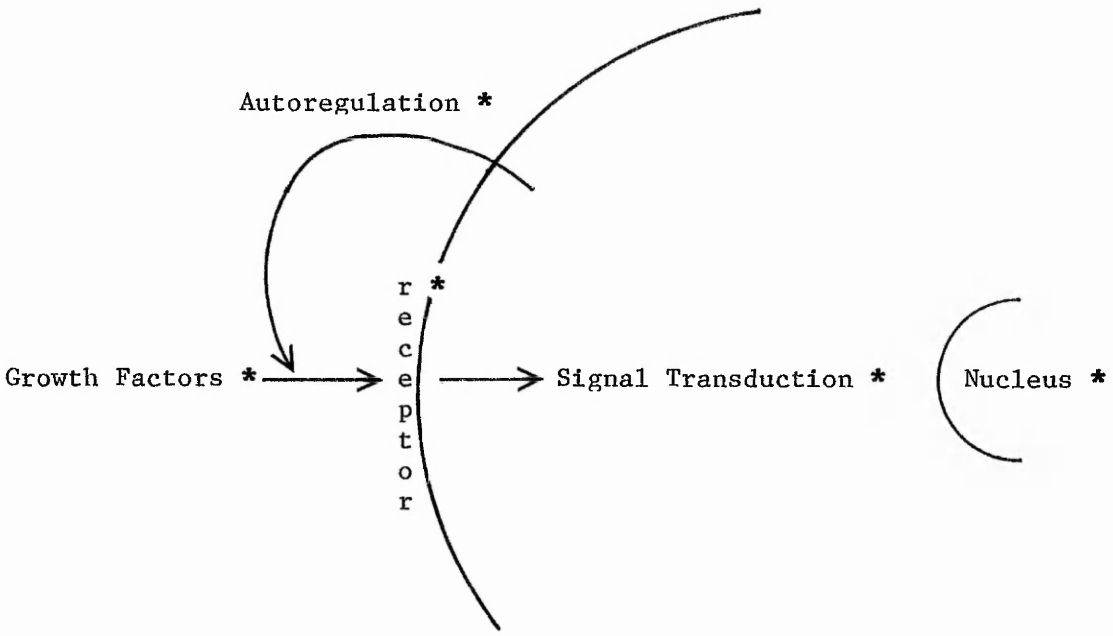
The issue of the relationship between tissue transglutaminase activity and the various forms and stages of cancer has become increasingly complex and controversial in recent years. The mass of experimental data available concerning transglutaminase and tumour progression, as well as the complex nature of carcinogenesis itself, necessitate the introduction of the topic in stages, following the multistage model of carcinogenesis (Weinstein et al, 1984; Parade and Weinberg, 1984).

1.3.5.1 Initiation.

Qualitative or quantitative disturbances of the proto-oncogenes encoding elements of the growth control network can lead to uncontrolled cell growth (Fig.1.3). As yet, none of the transglutaminases have been directly linked to any of the known oncogenes, however, Yuspa et al, 1985, reported that keratinocytes containing an activated ras oncogene are resistant to Ca^{2+}-induced differentiation, with fewer cells undergoing keratinisation, showing a tentative connection between v-ras^{H} and transglutaminase.

Birckbichler et al, 1981, proposed that tissue transglutaminase might be part of a negative control system, with low transglutaminase activity and cross-link levels allowing cell cycling to occur in transformed fibroblasts whilst high levels of cross-link and transglutaminase activity were found in the normal resting fibroblast. Other groups of workers have also observed low levels of transglutaminase in cultured transformed cells when compared to their non-transformed counterparts (Birckbichler et al, 1977; Birckbichler

Fig. 1.3 : Schematic representation of the growth control network governing cell proliferation.



* indicates possible places of malfunctioning derived from alterations to oncogene products.

and Patterson, 1978; Davies et al, 1984; Fesus et al, 1985). Birckbichler et al, 1977, also demonstrated reductions in the protein crosslink $\epsilon(\gamma$-glutamyl)lysine in transformed cells and these workers have since proposed that transglutaminase catalysed $\epsilon(\gamma$-glutamyl) lysine formation is important to the normal functioning of non-proliferating cells where the enzyme is thought to regulate membrane architecture (Birckbichler et al, 1978).

1.3.5.2 Promotion.

Tumour promoting agents appear to act by increasing cell proliferation, altering cellular phenotype or inducing genomic changes including the enhancement of malignant transformation (Weinstein, 1981). Phorbol ester type tumour promotors induce epidermal transglutaminase activity in epidermal basal cells (Yuspa et al, 1980; Yuspa et al, 1983). Cultured fibroblasts treated with phorbol esters also show increased levels of transglutaminase activity, with an increase in the amount of membrane associated transglutaminase (Fesus et al, 1985).

Conversely, Birckbichler et al, 1976, demonstrated reductions in transglutaminase activity associated with 3'-methyl-4-dimethylaminoazobenzene induced hepatomas and Novikoff hepatomas as opposed to normal liver, they also noted that the subcellular distribution of transglutaminase became more particulate in the tumours. Similar reductions in transglutaminase levels have also been reported by other workers. Vanella et al, 1983, observed decreases in the amount of cytosolic transglutaminase activity present in Yoshida ascites tumour cells and in ascites tumour bearing liver compared to

normal liver; Roch et al, 1987, noted that transglutaminase activity decreased sequentially from normal colon through benign colon to malignant colon carcinoma. Workers in this laboratory have shown similar decreases from normal liver through tumour bearing liver to hepatocellular carcinoma (Barnes et al, 1984, 1985; Hand et al, 1988). Additionally, Barnes et al reported an apparent redistribution of activity to the particulate fraction, whilst Hand et al, 1988, clarified this phenomena by demonstrating that the loss in activity was due to a specific decrease in cytosolic transglutaminase activity with no alteration in the particulate associated activity. In 1990, Hand et al further confirmed decreased transglutaminase activity in hepatocellular carcinomas by demonstrating reductions in the level of $\epsilon(\gamma$-glutamyl)lysine in the tumours compared to normal liver.

Other potentially important substrates of transglutaminase are the polyamines putrescine, spermine and spermidine (Folk, et al, 1979; Conrad, 1985), thought to play an important role in cell proliferation and differentiation (Chan et al, 1981; Pegg, 1986). Many studies have shown that proliferative stimulation, by hormones, carcinogens , drugs and tumour promotors, leads to the accumulation of polyamines (Janne et al, 1978; Scalabrino et al, 1978; Matsui and Pegg, 1982). Using experimental animal tumours, several groups have produced evidence for the accumulation of polyamines in tumours (Williams-Ashman et al, 1972; Noguchi et al, 1976; Kallio et al, 1977; Janne et al, 1978; Russell and Durie, 1978; Williams-Ashman and Canellakis, 1979; Kaminski, 1983; Hand et al, 1985, 1987). Since Birckbichler et al, 1980 and 1981, have linked alterations to transglutaminase activity with cell growth and proliferation, it is tempting to hypothesise that the observed accumulation of polyamines in cells stimulated to proliferate

and in tumours may be due to decreases in transglutaminase activity.

1.3.5.3 Progression.

Work in this laboratory, with chemically induced rat sarcomas, has indicated that the growth of metastatic tumours is accompanied by a decrease in the amount of transglutaminase activity present (Barnes et al, 1984,1985; Hand et al, 1987). Comparison of 'early stage' tumours with 'late stage' tumours known to have metastasised demonstrated a 50 - 80 % loss of activity; similar comparisons between 'early' and 'late' stage tumours from a tumour line known to be non-metastatic showed no significant alteration in the level of activity (Barnes et al, 1984, 1985). Evidence supporting this difference in the behaviour of non-metastatic and metastatic tumours, with respect to transglutaminase activity, came from Delcros et al, 1986. Using cloned cell lines derived from a rat rhabdomyosarcoma, these workers found that the higher the metastatic potential of the cell line, on intravenous injection, the lower the exhibited level of transglutaminase activity. This indicated an inverse correlation between tissue transglutaminase activity and the ability of tumour cells to extravasate and form metastases.

1.4 The transglutaminase gene.

Several reports emerged in 1987 on the partial sequencing, using cDNA technology, of the genes for tissue transglutaminase and the a subunit of the plasma transglutaminase Factor XIII (Stein et al,

1987; Ikura et al, 1987; Grundman et al, 1987; Ichinose and Davie, 1987). By Southern hybridisation, Stein et al could only demonstrate the presence of one gene for tissue transglutaminase in retinoic acid stimulated mouse macrophages, for which the mRNA was approximately 4.2 kb in size. Ikura et al obtained a cDNA from which they could predict approximately 60% of the primary structure of the tissue transglutaminase from guinea pig liver. They could not locate any regions showing the 'E-F hand' structure typical of high affinity calcium binding sites, but two regions were located that were rich in glutamic acid residues and they proposed these as two potential calcium binding sites. They also found a region within the amino acid sequence that corresponded to the active site sequence of Tyr-Gly-Gln-Cys-Trp, predicted by Folk and Cole, 1966. Working on the a subunit of Factor XIII from human placenta, Grundman et al, 1987, deduced that the gene encoding the enzyme consisted of 3905 base pairs, of which 1625 nucleotides made up a 3' non-coding region and poly(A) sequence with at least 84 base pairs representing a 5' non-coding region. The actual reading frame coded for 732 amino acids. These results were comfirmed by Ichinose and Davie, 1988, who also noted the presence of an activation peptide of 37 amino acids, two putative Ca^{2+} binding regions and an active site sequence of Tyr-Gly-Gln-Cys-Trp identical to that of the tissue transglutaminase. Ikura and co-workers went on to suggest that genetic polymorphism might exist for guinea pig liver transglutaminase (Ikura et al, 1988); that the amino terminal of the guinea pig liver transglutaminase is post-translationally acetylated, whilst the carboxy terminal undergoes no post-translational modification (Ikura et al, 1989a); and, by producing recombinant tissue transglutaminase in E.coli, showed that N-acetylation was not necessary

for the catalytic functioning of the enzyme (Ikura et al, 1989b). Takahashi et al, 1986, demonstrated that the catalytic a subunit of Factor XIII was also N-acetaylated and that, since the carboxy terminal is heterogenous, that this might also undergo post-translational modification. In 1988, Ichinose and Davie reported that the gene for Factor XIII contained 15 exons separated by 14 introns, and that the Ca^{2+} binding sites, thrombin cleavage site and the active site were all encoded by separate exons. Further, they showed, by restriction digests, that polymorphisms exist in the gene that might be helpful in studying various normal and abnormal genes. The mRNA coding for rabbit epidermal transglutaminase has also been isolated, it is approximately 3.6 kb in size, appears to be negatively regulated by retinoic acid and is distinct from tissue transglutaminase mRNA (Floyd and Jetten, 1989). Deduction of the amino acid sequence of human endothelial and mouse macrophage tissue transglutaminase from cDNA, has given a molecular weight of approximately 77,250 and 76,700 D respectively (Gentile et al, 1990). In the human, a single open reading frame of 2061 nucleotides was found, with a 3´ non-coding region of 1058 nucleotides containing no poly(A) sequence. The open reading frame for the mouse was found to be 2055 nucleotides, and again a 3´ untranslated region was found, this consisted of 1400 nucleotides and a 3´ poly(A) sequence. Homology between the 3´non-coding regions from the two species was low at approximately 40%. These workers also concluded that only one tissue transglutaminase message exists in cells, since Northern blot analysis produced only one band of approximately 3.6kb and transcription of the human cDNA, ´in vitro´, produced only one transglutaminase of 85,000 D (Gentile et al, 1990). They also concluded that, since the ´in vitro´ transcribed enzyme was an active

transglutaminase, post-translational modification of the enzyme was not necessary to bestow catalytic properties, confirmimg the work of Ikura et al, 1989b.

1.5 Induction of tissue transglutaminase.

In 1982, two groups of workers (Scott et al, 1982; Yuspa et al, 1982), noted that stimulation of melanoma cells, chinese hamster ovary cells and mouse epidermal cells, by retinoic acid, led to an increase in tissue transglutaminase activity. Since then, several groups have reported the induction of tissue transglutaminase activity by retinoids and their analogs (Moore et al, 1984; Davies et al, 1985; Lichti et al, 1985; Poddar and Davies, 1985; Moore and Davies, 1985; Rubin and Rice, 1986; Chiocca et al, 1988; Nara et al, 1989;). Stimulation of the leukaemic cell line HL60 with DMSO or retinoids causes their differentiation into myeloid cells, whilst stimulation of the cells with 12-O-Tetradecanoylphorbol-13-acetate (TPA) produces monocytes. Both retinoid and TPA stimulation of these cells induces tissue transglutaminase activity, however cells stimulated to differentiate into myeloid cells by DMSO show no alteration to their level of tissue transglutaminase activity (Maddox and Haddox, 1985). Therefore tissue transglutaminase induction is a consequence of stimulation, not an effect of differentiation. Moore et al, 1984, demonstrated the blocking of retinol induced tissue transglutaminase expression by actinomycin D, thereby indicating that retinoids were acting to increase gene expression. Further evidence to support this theory came from Murtaugh et al, 1986, and Davies et al, 1987, who demonstrated increases in the level of tissue transglutaminase mRNA

after retinol induction, suggesting an increase in the transcriptional activity of the gene; and from Chiocca et al, 1988, who found an accumulation of tissue transglutaminase mRNA after retinoic acid stimulation, independent of concurrent protein synthesis,
and suggested that a rapid increase in transcription of the tissue transglutaminase gene was occurring. More than one pathway for retinoic acid stimulation has been proposed (Poddar and Davies, 1985), since some analogs of retinoic acid induce transglutaminase expression in macrophages but not in myeloid leukemic cells. Various workers have also demonstrated the potentiation of the retinoic acid induction of tissue transglutaminase activity by the addition of cAMP between 24 and 48 hours after the addition of retinoic acid (Davies et al, 1985; Murtaugh et al, 1986; Maddox and Haddox, 1988); addition of cAMP in isolation has no effect on transglutaminase activity. These findings suggest that the retinol induced enhancement of transglutaminase activity is at least a two stage event, with a secondary stage potentiated by cAMP.

In epidermal cells, retinoic acid inhibits epidermal transglutaminase expression (Thacher et al, 1985; Jetten and Shirley, 1986; Rubin and Rice, 1986) whilst inducing the expression of cytosolic tissue transglutaminase (Yuspa et al, 1982; Lichti et al, 1985, Rubin and Rice, 1986; Lichti and Yuspa, 1988). Further, retinoic acid inhibits the induction of terminal differentiation and epidermal transglutaminase, by the phorbol ester TPA, leading to the suggestion that retinoic acid 'reprogrammes' epidermal cells by altering their responce to differentiation signals (Lichti and Yuspa, 1988).

Other substances have also been shown to increase transglutaminase activity. Novogrodsky et al, 1978, first indicated the

rapid (10 - 30 minutes) stimulation of transglutaminase activity in lymphocytes by phytomitogenic agents. Epidermal growth factor also causes a rapid (2 - 30 minutes) increase in the activity of a soluble transglutaminase within epidermal carcinoma cells (Dadabay and Pike, 1987). This increase is not a result of the induction of transglutaminase synthesis as cycloheximide treatment has no inhibitory effect, instead it suggests an activation of the enzyme already present in the cell. The rapid increases in transglutaminase activity in responce to mitogens noted by these workers may mean that transglutaminase is important in the mediation of the early effects of growth factors. Stimulation of transformed Wl-38 fibroblasts and PC12 pheochromocytoma cells by the differentiation promotor sodium butyrate causes growth arrest and morphological changes, and also leads to the induction of tissue transglutaminase (Birckbichler et al, 1983; Byrd and Lichti, 1987; Lee et al, 1987). This regulation of tissue transglutaminase is again thought to be at the pre-translational level, since the rate of transglutaminase synthesis and the level of transglutaminase mRNA increase (Lee et al, 1983). In contrast, nerve growth factor stimulation of PC12 cells, although causing growth arrest, does not induce transglutaminase activity (Byrd and Lichti, 1987), suggesting that the enzyme is not involved in growth regulation per se, rather in certain morphological changes accompanying differentiation.

1.6 Summary.

The precise role of tissue transglutaminase, despite a considerable number of investigations utilising various models, still remains undefined. It is, however, apparent that the enzyme is capable of modifying both membrane and cytoskeletal proteins, and its activity can therefore be implicated in the modification of membrane structure and function. Numerous reports also link transglutaminase activity with cellular development, from embryogenesis, through cell activation and differentiation, to cell senescence. Further it is apparent that inappropriate levels of transglutaminase activity, either hyper or hypo, may be associated with pathological conditions such as the increase in protein crosslinking in cataract formation or the reduced competancy of the cytoskeleton in neoplasia.

The aim of this thesis is to investigate further the reductions in transglutaminase activity that are associated with tumour progression and metastasis. The following four models will be used in order to elucidate the role, if any, that the enzyme plays in the processes involved in tumour progression :-

1) cultured variant cell lines, derived from a herpes simplex virus transformed hamster fibrosarcoma, showing differing metastatic potential 'in vivo', with a non-transformed hamster fibroblast cell line as a control;

2) the above variants, grown 'in vivo';

3) cultured metastatic variants of the B16 mouse melanoma, with the melanocyte cell line Melan A as a control;

4) metastasising and non-metastasising chemically induced, transplantable, rat sarcomas.

2: MATERIALS AND METHODS.

2.1 MATERIALS.

2.1.1 Abbreviations.

DMEM	-	Dulbeccos modified eagles medium
DMSO	-	dimethylsulphoxide
DNA	-	deoxyribonucleic acid
DNAse I	-	deoxyribonuclease I
DTT	-	dithiothreitol
EDTA	-	ethylenediaminetetraacetic acid
EGTA	-	ethylene glycol-bis(β-aminoethyl ether) N,N,N´,N´-tetraactic acid
FPLC	-	fast protein liquid chromatography
Goat anti-gplt	-	affinity purified anti-guinea pig liver transglutaminase raised in goat
HBSS	-	Hanks balanced salt solution
HEPES	-	N-2-hydroxyethylpiperazine-N´-2-ethane sulphonic acid
HPLC	-	high performance liquid chromatography
HRP	-	horse-radish peroxidase
LAP	-	leucine amino peptidase
OPA	-	o-phthalaldehyde
PAGE	-	polyacrylamide gel electrophoresis
PBS	-	phosphate buffered saline
PMSF	-	phenylmethylsulphonylfluoride
Rabbit anti-rlct	-	anti-rat liver cytosolic transglutaminase raised in rabbit

SDS	-	sodium dodecyl sulphate
TBS	-	Tris buffered saline
TCA	-	trichloroacetic acid
TEMED	-	N,N,N′,N′-tetramethylethylenediamine
TMB	-	3,3,5,5-tetramethyl benzidine
Tris	-	Tris(hydroxymethyl)-aminomethane

2.1.2 Chemicals and Equipment.

The chemicals used for this research were purchased from the following sources :-

Amersham International PLC., Little Chalfont, Bucks. : all radiochemicals.

Boehringer Corporation (London) Ltd., Lewes, East Sussex : standard DNA from calf thymus.

British Drug Houses Ltd., Poole, Dorset : electran grade :- acrylamide, ammonium persulphate, N,N′-methylenebisacrylamide, SDS, TEMED, PAGE blue 83 : analar grade :- all other chemicals and solvents not specifically mentioned.

Clay-Adams, (A Division of the Becton Dickinson Company), Parsippany, New Jersey, USA. : Autoclip 9mm (stainless steel wound clips).

Fahrenheit Laboratory Supplies (Midlands) Ltd., Sandiacre, Notts.

Flow Laboratories, Rickmansworth, Herts. : BHK-21 cell line.

ICI PLC., Pharmaceuticals Division, Macclesfield, Cheshire : Hibitane.

ICN Biomedicals, High Wycombe, Bucks. : Cellagen TM CD24 Discs.

Millipore / Waters, Watford, Beds. : Nova-Pak C18 column, 0.22um

filters (60, 20 and 10 mm diameter).

Pharmacia / LKB Ltd., Uppsala, Sweden : Aldolase (protein molecular weight marker), 1ml Mono-Q anion-exchange column, PD-10 columns, Q-sepharose Fast Flow, Ultrogel ACA 44, 26.5ml UltroPac TSK SW 3000 gel filtration column.

Pierce, Chester, Cheshire : Picobuffer IV system.

Serva, Cambridge Bioscience, Cambridge : $\epsilon(\gamma$-glutamyl)lysine.

Sigma Chemical Company, Poole, Dorset : Anti-species HRP conjugates, benzamidine, bovine serum albumin, Brij-35, 4-chloro-1-napthol, collagenase type VII, diphenylamine, dithiothreitol, DMSO, DNAse 1, HEPES, lectin-HRP conjugates, lipase type VII, Lubrol-PX, 2-mercaptoethanol, poly-l-lysine, putrescine dihydrochloride, PMSF, sucrose, Trizma base, o-phthalaldehyde, protein molecular weight markers.

Centrifuges : (All centrifugation speeds in this thesis are described as g_{av}.)

Beckman, California, USA. :

Centrifuge	Rotor	RCF (g_{av})
1 : Ultracentrifuge	8x25 angle	71,000

MSE Scientific Instruments, Crawley, Sussex. :

1 : MSE Centaur	8x10 ml swingout (r_{av} = 12cm)	700
2 : MSE Microcentaur	-	7500
3 : MSE Hispin 21	8x50 ml angle	

CO_2 Incubator : Flow Laboratories, Rickmansworth, Herts. : Model IR 1500.

Conductivity Meter : Corning Ltd., Essex. : Model 220.

Data Aquisition : Nelson Analytical, Cupertino, California, USA : 900 series interface box; data aquisition software, serial no. 00001418.

Data Storage : Walters International, High Wycombe : Personal computer.

Electrophoresis Apparatus : Biorad Ltd., Hemel Hempstead, Herts : Protean II Minigel vertical system.

: Pharmacia / LKB, Uppsala, Sweden. : Multiphor II Dry Blot.

ELISA Plate Reader : Titertek, Flow Laboratories, Rickmansworth, Herts : Model

Fluorimeters : Perkin Elmer, Beaconsfield, Bucks. : Models 1000M and LS-1.

HPLC Apparatus : Millipore / Waters, Watford, Herts : M-45, 510 and 6000 pumps; 660 gradient controller.

: Beckman, California, USA. : System Gold pumps, controller, and data aquisition.

Liquid Nitrogen Store : Union Carbide, Jencons (Scientific) Ltd., Leighton Buzzard, Beds. : Model 35HC.

Liquid Scintillation Spectrophotometer : Canberra Packard, Pangbourne, Berks. : Model A 300 CD.

Microscopes : Olympus, : Model CK2 inverted microscope.

: Leitz, : Dialux fluorescent microscope.

pH Meter : Corning Ltd., Essex. : Model 130.

Sonicator : MSE Scientific Instruments, Crawley, Sussex. : Soniprep 150.

Spectrophotometer : Pye-Unicam, Cambridge. : Model SP6-400.

Sterile Cabinets : Flow Laboratories, Rickmansworth, Herts. : Gelaire BSB 4A; Hepaire Class 1.

Ultra-Turrax : Copley Scientific Instruments and Equipment, Nottingham.

2.1.3 Animals.

For the growth of HSV-2 induced fibrosarcoma cell lines 'in vivo' male Syrian hamsters were used.

For the growth of chemically induced, transplantable sarcomas 'in vivo' AS rats of both sexes were used.

2.2 'IN VITRO' TECHNIQUES.

2.2.1 Cell Culture.

Hamster fibroblast cell lines, mouse melanoma cell lines and mouse melanocytes were grown at 37°C in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 5% (v/v) horse serum, and maintained in a 5% (v/v) CO_2 atmosphere. For passage, cells grown in T75 flasks (Falcon) were released with trypsin/EDTA (0.5g/l trypsin, 0.2g/l EDTA in Ca^{2+} and Mg^{2+} free HBSS), pelleted by centrifugation in an MSE Centaur 1 bench top centrifuge at 1500rpm for 5min, resuspended in DMEM and split 1 in 5 (approximately $1x10^6$ cells) then reseeded into T75 flasks with 15ml of fresh medium. With this regime, cells required splitting once a week and remained healthy. Surplus cells at passage were frozen down at -120°C (section 2.2.2). For experiments, 2×10^6 cells from a subconfluent T75 flask were seeded into T150 flasks (Falcon), grown in supplemented medium (30ml) for 60h and then left overnight in serum-free medium, this produced an average of 1.6×10^7 cells per flask. Cells for use in experiments were aspirated, then incubated for 5min with 5mM Tris-HCl pH7.4, 2mM EDTA to release them from the plastic and pelleted by centrifugation as for passage.

2.2.2 Cell Storage.

Cells were stored under nitrogen vapour (-120°C) at a density of 10^7/ml in 95% (v/v) horse serum, 5% (v/v) DMSO. This enabled cells from similar passage numbers to be used throughout.

2.2.3 Trypan Blue Exclusion Test For Cell Viability.

Samples from cell suspensions were mixed 1:1 with Trypan Blue solution (4 parts 0.2% (w/v) trypan blue, 1 part 4.5% (w/v) saline). Cells were counted in a hemocytometer, and the number of blue stained cells expressed as a percentage of the total number of cells.

2.2.4 Assessment of Cell Invasion 'in vitro'.

This procedure used a modification of the method of Repesh (1989). Sterile 24 well tissue culture plates were used to house sterile collagen membranes mounted on plastic stands (Cellagen TM CD24 Discs, ICN Biomedicals). Supplemented medium (0.5ml) was placed in each lower chamber and 0.5ml cell suspension (2×10^3 cells in supplemented medium) placed in each upper chamber. After 72h of incubation the number of cells adhering to each surface of the membrane was counted and expressed as a percentage of the original number of cells seeded.

2.2.5 Apoptotic Body Isolation (Schmidt et al, 1988).

Cells ($2 - 3 \times 10^7$) were released with 5mM Tris-HCl pH7.4, 2mM EDTA, collected by centrifugation (700g, 5min), resuspended in 1ml serum free DMEM to which was then added 100ul of a 20% (w/v) solution of SDS containing 2mM PMSF in 1% (v/v) final volume DMSO and 65uM DTT and heated for 5min at 100°C. After cooling, 10ul of a 1mg/ml solution of DNAse I was added, and the SDS insoluble bodies counted on a hemocytometer.

2.3 TUMOURS.

Two experimental tumour models were used, one induced 'in vivo' by carcinogens, the other derived from an 'in vitro' transformation of embryonic cells.

2.3.1 Passage of rat sarcoma lines.

Four transplantable rat sarcomas were used in this study. Details of their origins and growth characteristics can be found in Moore, 1972. Briefly, their origins are :-

P7 osteosarcoma induced by intraperitoneal injection of ^{32}P.

P8 fibrosarcoma induced by intraperitoneal injection of ^{32}P.

MC3 fibrosarcoma induced by methylcholanthrene dose.

CC5 osteosarcoma induced by cupric-chelated-N-hydroxy-2-acetyl-amino-fluorene dose.

Propagation of sarcomas was by the subcutaneous implantation of freshly excised, non-necrotic, tissue into AS rats of either sex. Rats were anaesthetised with ether and a patch on their upper right flanks (15mm x 15mm) plucked or shaved and sterilised with Dispray 1 (Stuart Pharmaceuticals Ltd.). A small incision was made through the hairless skin, and a subcutaneous pocket (approx. 10mm x 3mm) formed using scissors. Freshly excised, non-necrotic, tumour tissue (approx. $2mm^3$) was inserted into this pocket and the incision closed with a stainless steel wound clip (Clay-Adams). Animals were checked for tumour growth at regular intervals, and sacrificed before, or in the case of time courses, at the first signs of, distress.

2.3.2 Propagation of hamster fibrosarcomas.

Five hamster fibrosarcomas, whose origins and characteristics have been described previously (Walker et al, 1982; Teale and Rees, 1987) were used. In brief, their origins are as follows :-

Parent Inactive HSV-2-333-2 induced 'in vitro' transformation of hamster embryo fibroblasts.

Met B
Met C
Met D
Met E
Met F

} Derived from lung nodules of animals whose primary parent tumour load had been resected.

Cultured cells from the parent cell line and four of its metastatic variants (Met B, D, E and F) were harvested following trypsinisation of confluent monolayers, washed three times in Hanks balanced salt solution (HBSS) and resuspended in HBSS at 10^5 cells per ml. Cell suspensions were assessed for viability by trypan blue exclusion, and only those preparations of more than 90% viability were used. Tumour propagation was effected by subcutaneous injection of 0.1 ml of cell suspension (10^4 cells) into Syrian golden hamsters. Animals were checked for tumour growth at regular intervals, and sacrificed before they became distressed.

2.3.3 Harvesting of tumour tissue.

Animals were sacrificed by cervical dislocation and non-necrotic tumour tissue removed for analysis. Prior to analysis, all tumour tissue was kept on ice.

2.3.4 Resection of tumours for evaluation of metastatic potential.

Developing tumours of approximately 10mm diameter, 12 days after transplantation, were resected. The tumour mass was excised, from animals anaesthetised with ether, and the skin around the excision loosened from the underlying musculature. Stainless steel wound clips (9mm) were used to close the wound, which was then sterilised with Dispray 1. Animals were closely monitored post-resection, those showing signs of respiratory distress or illness were sacrificed and examined for secondary tumours. All surviving animals were sacrificed at 7 weeks post-resection and examined for signs of metastatic spread. Metastatic nodules were removed from the lungs, samples ($3mm^3$) taken for storage at -120^oC, and the rest analysed for their transglutaminase content.

2.3.5 Detection of lung metastases.

To detect secondary tumour growth in animals whose primary tumour load had not been resected, the method of Wexler (1966) was employed. Indian ink (15% (v/v) containing 0.5% (v/v) ammonia solution) was used to inflate the lungs in situ via the trachea. The trachea was then ligated and the lungs removed. After washing in distilled water, the lungs were fixed in Feketes solution (58% (v/v) ethanol, 50ml distilled water, 8.7% (v/v) of a 40% (v/v) formaldehyde solution and 4.4% (v/v) glacial acetic acid). Metastases appeared white on a black background.

2.3.6 Collection of human breast tumour tissue.

Samples (~600mg) of human breast tumours, and 'normal' breast tissue, removed at surgery, were snap frozen in liquid nitrogen and stored at -120°C prior to analysis. For these samples I am indebted to the Pathology Dept. of The Royal Hallamshire Hospital, Sheffield, and the Histopathology Unit., of The City Hospital, Nottingham.

2.4 FRACTIONATION METHODS.

2.4.1 Tissue Homogenisation.

Tissue was kept on ice before, during and after homogenisation. All tissue homogenates were made up to 20% (w/v) after homogenisation.

Normal liver; hamster and rat tumours :-

Non-necrotic tissue was weighed and then washed in cold Buffer A (5mM Tris-HCl pH7.4, 2mM EDTA). Tissue was then minced in two volumes of cold homogenising buffer (0.25M sucrose, 5mM Tris-HCl pH7.4, 2mM EDTA, 1mM DTT, 5mM benzamidine, 1mM PMSF in DMSO to 1% (v/v) final volume) by use of a variable speed Ultra-Turrax with 1 x 30 sec burst at low speed (the blade was precooled to $4^{o}C$ in ice cold Buffer A). After addition of another two volumes of cold homogenising buffer, minced tissue was homogenised with five passes of a close fitting (clearance 0.25 - 0.30 mm) glass/teflon Potter-Elvehjem homogeniser.

Human tumours :-

Tissue was thawed on ice, weighed and washed in cold Buffer A. Samples (50 - 200 mg) were homogenised in two volumes of cold homogenising buffer containing 1% (w/v) lubrol-PX, 1μg/ml leupeptin and 1μg/ml pepstatin, using five passes of a microhomogeniser (homogenising took place in a Category I cabinet).

Cultured cells :-

Cells were washed twice with cold Buffer A, resuspended to 10^{7} cells/300μl in cold homogenising buffer containing 1% (w/v) lubrol-PX, and homogenised using five passes of a microhomogeniser.

2.4.2 Detergent Extraction of Transglutaminase.

This procedure is based on the methods of Chang and Chung (1986). Tissue, or cell suspension, was homogenised, as above, in cold homogenising buffer containing 1% (w/v) lubrol-PX and allowed to stand at 4°C for 45min. The homogenate was then centrifuged at 71,000g for 45min at 4°C, the resultant supernatant decanted and the pellet resuspended, by means of a precooled Ultra-Turrax, in an equivalent volume of homogenising buffer containing 1% (w/v) lubrol-PX. This procedure was repeated twice. The three resultant supernatants were then combined and dialysed for 1h at 4°C against two changes of 5mM Tris-HCl pH7.4, 2mM EDTA, 2mM DTT. Resuspension of the final pellet was in cold buffer A.

2.4.3 Fractionation into cytosol and 71,000g pellet.

Tissue homogenised in homogenising buffer was centrifuged at 71,000g for 45min at 4°C. The cytosolic supernatant was decanted and the pellet resuspended in an equivalent volume of cold homogenising buffer containing 1% (w/v) lubrol-PX, by means of an Ultra-Turrax, ready for detergent extraction as above.

2.5 ELECTROPHORESIS METHODS.

2.5.1 SDS-Polyacrylamide Electrophoresis.

The electrophoretic separation of reduced proteins was achieved using modifications of the discontinuous system described by Laemmli (1970). A Bio Rad vertical mini-gel unit was used with 0.5mm thick slab gels consisting of a 2.5% (w/v) acrylamide stacking gel and either a 6.5% (w/v) acrylamide or a 10% (w/v) acrylamide resolving gel.

Solutions :-

Electrode Buffer	0.025M Tris-HCl pH 8.3
	0.192M glycine
	1% (w/v) SDS
Sample Buffer	0.08M Tris-HCl pH 6.8
	2% (w/v) SDS
	10% (v/v) glycerol
	0.0024% (w/v) bromophenol blue

Gels :-

Acrylamide Solution	30% (w/v) Acrylamide
	1% (w/v) Methylene bis Acrylamide
2.5% Stacking gel	0.5ml Acrylamide solution
	2.0ml 0.25M Tris-HCl pH6.8
	1.5ml distilled water
	50.0ul 10% (w/v) SDS
	50.0ul 10% (w/v) $(NH_4)_2S_2O_8$
	5.0ul TEMED

6.5% Resolving gel	1.30ml Acrylamide solution
	2.24ml 1M Tris-HCl pH8.8
	6.05ml distilled water
	50.00ul 10% (w/v) SDS
	50.00ul 10% (w/v) $(NH_4)_2S_2O_8$
	5.00ul TEMED
10% Resolving gel	2.00ml Acrylamide solution
	2.24ml 1M Tris-HCl pH8.8
	1.72ml distilled water
	50.00ul 10% (w/v) SDS
	50.00ul 10% (w/v) $(NH_4)_2S_2O_8$
	5.00ul TEMED
Fixative and Stain	200ml Methanol
	40ml Glacial Acetic Acid
	200ml distilled water
	0.1% (w/v) Coomassie Blue
Destain	25ml Methanol
	35ml Glacial Acetic Acid
	440ml distilled water

2.5.2 Non-SDS-Polyacrylamide Electrophoresis.

The electrophoretic separation of non-reduced proteins was achieved using a Bio Rad vertical mini-gel unit with 0.5mm thick slab gels consisting of a 7.5% (w/v) acrylamide resolving gel.

Solutions :-

Electrode buffer	38mM glycine
	48mM Tris pH8.2
Sample buffer	0.08M Tris-HCl pH 6.8
	10% (v/v) glycerol
	0.0024% (w/v) bromophenol blue

Gels :-

Acrylamide Solution	33% (w/v) Acrylamide
	0.5% (w/v) Methylene bis Acrylamide
7.5% Resolving gel	6.75ml Acrylamide solution
	9.0ml 376mM Tris-HCl pH8.9
	11.25ml distilled water
	3ml 0.75% (w/v) $(NH_4)_2S_2O_8$
	20ul TEMED
Fixative and Stain	20ml Methanol
	50ml Glacial Acetic Acid
	225ml distilled water
	0.2% (w/v) PAGE Blue
Destain	40ml Methanol
	100ml Glacial Acetic Acid
	450ml distilled water

2.5.3 Western Blotting.

Electrophoretic transfer of protein from gels to nitrocellulose paper was performed using an LKB Multiphor II Dry Blot.

2.5.3.1 Transfer of Proteins From Reducing Gels.

Wicks for the anode and cathode (9 pieces of Whatman 3M filter paper per wick, cut to the size of the gel) and nitrocellulose paper (cut to gel size) were wetted by capillary action and then soaked for 10min in a 1/5 dilution of electrode buffer (2.5.1). A transfer unit was then set up as follows :-

Graphite anode, presoaked with distilled water

Anode wick

nitrocellulose

SDS PAGE Gel

Cathode wick

Graphite cathode, presoaked with distilled water.

Care was taken at all stages to avoid the entrapment of air bubbles and the contamination of any part of the transfer unit with extraneous protein. Transfer of protein was effected by passing a constant current from the cathode to the anode (0.75mA cm^{-2} area of gel for 50min, 0.6mA cm^{-2} area of gel for 20min).

2.5.3.2 Transfer of Proteins From Non-reducing Gels.

Electrode wicks (as 2.5.3.1) and nitrocellulose paper were wetted by capillary action and soaked in electrode buffer (section 2.5.2) containing 20% (v/v) methanol for 10min. The setting up of transfer units, and the transfer of proteins was then as above.

2.5.4 Immunodevelopment of Western Blots.

Western blots were incubated in Blocking solution for 1h at room temperature or overnight at 4°C, with gentle shaking, and then rinsed in TBS containing 0.1% (v/v) Tween-80. The following protocol was then used to immunoprobe the bound protein :-

Primary antibody, diluted 1/1000 with blocking solution, incubate at 4°C overnight with gentle shaking.

Wash in TBS, 3 x 10min at room temperature.

Second antibody, commercial anti-species biotin conjugate diluted as recommended with TBS containing 1% (w/v) dried milk powder, incubate at room temperature for 2h with gentle shaking.

Wash in TBS, 3 x 10min at room temperature.

Incubate with commercial peroxidase labelled avidin, diluted as recommended with TBS, for 30min at room temperature with gentle shaking.

Wash in TBS, 3 x 10min at room temperature.

Rinse with distilled water and equilibrate with PBS.

Incubate with chloronapthol reagent at room temperature for 15min then stop reaction by washing thoroughly with distilled water.

Solutions :-

Blocking solution	100mM Tris-HCl pH 9.0
	150mM NaCl
	0.05% (v/v) Tween-80
	1% (w/v) dried milk powder
TBS	50mM Tris-HCl pH 7.4
	200mM NaCl

PBS (0.15M pH7.4)	8.00g NaCl
	0.20g KCl
	1.15g Na_2HPO_4
	0.20g KH_2PO_4
	1.01 distilled water
Chloronapthol reagent	20mg 4-chloro-1-napthol in 2.5ml DMSO
	47.5ml PBS
	15.0ul 30% (v/v) H_2O_2

2.6 TRANSGLUTAMINASE ASSAYS.

2.6.1 Transglutaminase activity.

Transglutaminase activity was quantitated in an 'in vitro' assay by measuring the rate of incorporation of radiolabelled putrescine into N,N'-dimethylcasein, using the filter paper assay of Lorand et al, 1972.

For accurate assessment of activity, [1,4-^{14}C]-putrescine was used, however for determining activity in column eluents, [1,4(n)-^{3}H]-putrescine was used as this provided increased sensitivity.

Activity was expressed in units, one unit being the amount of putrescine (nanomoles) incorporated into 5mg of N,N'-dimethylcasein per hour.

2.6.1.1 [^{14}C]-Putrescine Assay.

Reaction vials (0.5ml microcentrifuge tubes) were incubated at 37^{o}C and contained the following, in a volume of 25μl :-

Tris-HCl	55.0mM
N,N'-dimethylcasein	10.0mg/ml
DTT	7.7mM
[1,4-14C]-Putrescine (3.97mCi/mmol)	2.4mM
Calcium chloride	6.0mM
or	
EDTA (control)	20.0mM

(all solutions were at pH 7.4 at 37^{o}C)

Initiation of the reaction was by the addition of sample

(25μl) to the reaction mixture. At 2, 5 and 10 min, 10μl aliquots were removed, spotted onto filter paper squares (1cm^2, lightly marked with pencil, section 2.6.1.4) and placed in ice-cold 10% (w/v) TCA to stop the reaction and to precipitate protein onto the filter paper. As a measurement of total counts in the assay (for the estimation of counting efficiency see section 2.6.1.6), 10μl of the remaining reaction mixture from each vial was spotted onto a filter paper square and air dried.

Control incubations were performed in which $CaCl_2$ was replaced by 10mM EDTA, and although these gave no rate of incorporation, they did give background counts of between 50 - 110 cpm.

2.6.1.2 [^{3}H]-Putrescine Assay.

This assay follows the same procedures as for the [^{14}C]-putrescine assay detailed above, except that duplicate aliquots were taken after 1h incubation. Background EDTA levels were in the range 60 - 180 cpm. The reaction mixture (in a total volume of 18μl) was as follows :-

Tris-HCl	55.0mM
N,N'-dimethylcasein	10.0mg/ml
DTT	7.7mM
[1,4(n)-^{3}H]-Putrescine (11.1Ci/mmol)	15.2μM
Calcium chloride	6.0mM
or	
EDTA (control)	20.0mM
(all solutions were at pH 7.4 at 37^{o}C)	
Sample	12.0μl

2.6.1.3 Filter Papers.

For the transglutaminase assays, samples for scintillation counting were precipitated onto 1 cm square filter papers (Whatman 3MM) presoaked in 100mM EDTA and 1% (w/v) methylamine, then dried in an oven prior to use, and lightly marked with pencil for identification. After washing to remove unbound label, filter papers were placed in plastic inserts and covered with 2ml of Optiphase Highsafe for counting.

2.6.1.4 Washing of filter papers.

The washing of filter papers was achieved by placing them in a perforated beaker (250ml) suspended within a stirred beaker (500ml) that was kept on ice. A minimum volume of 5ml of washing solution per filter paper square was maintained throughout. The following washing procedure was used :-

1 x 10% (w/v) TCA, 1% (w/v) methylamine	10 min
3 x 5% (w/v) TCA, 1% (w/v) methylamine	- 15 min
1 x 1:1 Acetone:Ethanol	- 15 min
1 x Acetone	- 5 min

Filter papers were then air dried overnight and counted.

2.6.1.5 Determination of Radioactivity.

The radioisotopes ^{14}C and ^{3}H were detected by scintillation counting in a Packard A 300 CD liquid scintillation spectrophotometer. The channels are as shown in table 2.1.

Table 2.1

ISOTOPE	CHANNEL A (Kv)	CHANNEL B (Kv)
^{14}C	0 - 156	4 - 156
^{3}H	0 - 19	2 - 19

2.6.1.6 Determination of Counting Efficiency.

Counting efficiency for filter papers was calculated by spotting known amounts of radioactive reaction mixture onto filter papers which were then counted as previously described. All squares in an assay were assumed to have equal counting efficiency. In addition, the Packard quench indication parameter and the channels ratio (B/A) were used to monitor for any unexpected quenching which occurred in the samples. Any results that were affected in this way were repeated. In general an efficiency of 75-85% was found for all ^{14}C samples and one of 30 - 40% for ^{3}H.

2.6.2 $\epsilon(\gamma$-glutamyl)lysine Analysis.

Endogenous transglutaminase activity was assessed by the analysis of the level of $\epsilon(\gamma$-glutamyl)lysine in tissue and cell homogenates. A direct approach was used for quantitation necessitating the isolation of the isodipeptide from crosslinked proteins. This isolation was achieved by proteolytic digestion since the integrity of the isodipeptide is resistant to the proteolytic action of a variety of

enzymes. The following protocol was used for the direct estimation of ϵ(γ-glutamyl)lysine :-

Exhaustive proteolytic digestion

Prepurification

Separation and quantitation by reverse phase HPLC

2.6.2.1 Proteolytic Digestion For ϵ(γ-glutamyl)lysine Analysis.

Protein (approximately 10mg) was precipitated by the addition of TCA to a final concentration of 10% (w/v), centrifuged for 30sec in a microcentrifuge and the resultant pellet washed as follows :-

1 x 10% (w/v) TCA

3 x diethylether : ethanol 1 : 1

3 x diethylether.

The final pellet was dried thoroughly and rehydrated in 50μl H_2O, 950μl 0.1M $(NH_4)_2CO_3$ pH8 at 32°C (1 crystal of thymol was added to each sample to prevent bacterial decay). Various proteolytic enzymes were then added as in the following protocol :-

0.1mg subtilisin, incubate at 32°C for 12h with gentle shaking, repeated twice at 10h intervals

0.15mg pronase, incubate at 32°C for 12h with gentle shaking, then at 100°C 15min to inactivate pronase

90μl of activated leucine aminopeptidase, 75μl of activated prolidase and $MgCl_2$ to a final concentration of 5mM, incubate at 37°C for 10h, repeated once

Digest adjusted to pH 6.75 - 7.00

0.2mg carboxypeptidase Y, incubate at 30°C for 12h.

Activation of leucine amino peptidase (LAP) :-

10µl 50mM $MnCl_2$

90µl 10mM Tris-HCl pH8

100µl LAP (22.75U)

Incubate at 37°C for 2 - 3 h.

Activation of prolidase :-

20ul 50mM $MnCl_2$

80ul 10mM Tris-HCl pH8

80ul distilled water

20ul prolidase (38.6U)

Incubate at 37°C for 2 - 3 h.

Samples were placed overnight in a dessicator, under vacuum, containing one beaker of 0.5N NaOH and one beaker of 0.5N H_2SO_4, to remove $(NH_4)_2CO_3$.

2.6.2.2 Prepurification by Cation-exchange.

In cellular proteins, the amount of isodipeptide may be as low as 0.1 mole per 10^6g of protein. Therefore it is advantageous to remove those amino acids that interfere with the resolution of the isodipeptide peak obtained by HPLC.

A 5ml column of Dowex 50 x 4 - 400 resin (in the hydrogen form), pre-equilibrated in Milli-Q pure water pH3 with HCl, was utilised for the separation of $\epsilon(\gamma$-glutamyl)lysine from the amino acids Tyrosine, methionine and leucine. Digested sample (100µl, approximately 1mg protein) was applied to the column and eluted with a 40ml linear gradient of 0.06M ammonium formate pH2.5 to 0.3M ammonium

formate pH3.4. Fractions (5ml) were collected and placed overnight in a dessicator, under vacuum, containing one beaker of 0.5N NaOH and one beaker of 0.5N H_2SO_4, to remove $(NH_4)_2CO_3$ before being freeze dried. The dipeptide was located in fraction 2 and occasionally 3 as well. Recovery of dipeptide from the cation-exchange column (90%) was estimated by applying a known amount of commercial ε(γ-glutamyl)lysine standard (100nmoles) and quantitating the amount in the relevant fractions as described in section 2.6.2.3.

2.6.2.3 Quantitation of ε(γ-glutamyl)lysine.

Quantitation of the isodipeptide was achieved using reverse phase HPLC (2.8.3) and was verified, on random samples, using an amino acid analyser (2.8.4). Peaks were verified as being ε(γ-glutamyl) lysine by the addition of known amounts of ε(γ-glutamyl)lysine (Serva), and were quantitated by the subtraction of unspiked peak from spiked peak and by reference to the peak heights of known amounts of ε(γ-glutamyl)lysine (Serva).

2.6.3 Quantitation of Transglutaminase Antigenic Protein.

The presence of transglutaminase protein (antigen) in column eluent fractions was established using an enzyme linked immunosorbent assay (ELISA) utilising poly-l-lysine and glutaraldehyde to bind protein to the plastic assay plate. Quantitation of transglutaminase protein was performed using a sandwich ELISA.

2.6.3.1 Sandwich ELISA Protocol.

The determination of the dilutions used in this assay are described in section 4.2.2.

Microtitre assay plates (96 wells, U bottomed, Falcon) were coated with 50µl per well of affinity purified goat anti-guinea pig liver cytosolic transglutaminase antibody (goat anti-gplt) (courtesy of Dr.P.J.Davies, Dept. of Pharmocology, University of Texas Medical School, Houston, TX., U.S.A.) diluted 1/4000 in PBS. Plates were covered and left overnight at 4°C before being washed 3 times with PBS. Unbound sites on the plastic were then blocked for 1h at 37°C with 2% (w/v) dried milk powder in PBS (250µl per well). After washing (3 times with washing buffer), samples were added in triplicate (50µl per well), and a standard curve of purified guinea pig liver cytosolic transglutaminase (courtesy of Dr.D.Hand, Dept. of Life Sciences, Nottingham Polytechnic, Nottingham, U.K.) diluted in PBS (50µl per well ranging from 350 to 2.5 ng per well) set up in triplicate. Plates were incubated for a further 2h at 37°C and then washed 3 times with washing buffer. Rabbit anti-rat liver cytosolic transglutaminase (rabbit anti-rlct) (courtesy of Dr.D.Hand, Dept. of Life Sciences, Nottingham Polytechnic, Nottingham, U.K.), diluted 1/1000 in PBS containing 2% (w/v) dried milk powder, was added (50µl per well) and the plates covered and left overnight at 4°C. After washing (3 times with washing buffer), 50µl per well of a 1/1000 dilution of commercial (Sigma) anti-rabbit horse-radish peroxidase (HRP) conjugate in PBS containing 2% (w/v) dried milk powder, was added and the plates incubated for 2h at 37°C. Plates were then washed 3 times with washing buffer and twice with distilled water, before adding HRP substrate (100µl per well).

Colour was allowed to develop at room temperature for 20min, the reaction was then stopped by the addition of 50ul per well of 2.5M H_2SO_4 and plates read at 450nm in a Titretek Plate Reader.

Controls were included as follows :-

Control serum for goat anti-gplt

PBS for sample

Control serum for rabbit anti-rlct.

2.6.3.2 Poly-l-lysine ELISA.

Microtitre plates (96 wells, U bottomed, Falcon) were incubated at room temperature for 1h with 50µl per well of 1mg/ml poly-l-lysine in PBS. Plates were washed with PBS, 50µl sample added per well and left at room temperature for 30min. Ice cold glutaraldehyde (0.5% (v/v) in PBS) was added to each well and the plates left for a further 30min at room temperature. After washing 3 times with PBS, plates were blocked for 1h at room temperature with PBS containing 2% (w/v) dried milk powder and 100mM glycine (250µl per well) and then for a further 30min at room temperature with PBS containing 2% (w/v) dried milk powder (250µl per well). After washing 3 times with washing buffer, rabbit anti-rlct diluted 1/1000 in PBS containing 2% (w/v) dried milk powder, was added (50µl per well) and the plates covered and left overnight at $4^{o}C$. The remainder of the sandwich ELISA protocol was then followed.

Controls were included as follows :-

PBS for sample

Control serum for rabbit anti-rlct.

Solutions :-

PBS (0.15M pH7.4)	8.00g NaCl
	0.20g KCl
	1.15g Na_2HPO_4
	0.20g KH_2PO_4
	1.00l distilled water
Washing buffer	0.05% (v/v) Tween-80 in PBS
HRP substrate	20ml 0.1M sodium acetate-acetic acid pH6
	150ul TMB (10mg/ml in DMSO)
	25ul 3% (v/v) H_2O_2

2.7 CHEMICAL DETERMINATIONS.

2.7.1 DNA Estimation.

The method of Burton, 1956, was employed for the estimation of DNA. Ice-cold 10% (w/v) TCA (2.6ml) was added to 20% (w/v) tissue homogenate (0.4ml) and the resultant precipitate pelleted by centrifugation at 700gav for 10 min. The pellet was then resuspended in 10% (w/v) TCA (3ml) and recentrifuged. This pellet was then twice washed in 95% (w/v) ethanol, recentrifuged and the final pellet thoroughly drained.

DNA hydrolysis was performed by the addition of 5% (w/v) TCA (3ml) and incubation at 90^{o}C for 10min. The lysate was clarified by centrifugation, and 1ml added with vortexing to freshly prepared diphenylamine reagent (2ml) (1.5g diphenylamine, 100ml Analar glacial acetic acid, 1.5ml conc. H_2SO_4, 0.5ml of 16 mg/ml acetaldehyde) in acid washed glass tubes. Colour development was allowed to occur overnight, in the dark at room temperature prior to absorbance being read at 600nm.

A standard curve of calf thymus DNA in the range 10 - 100 μg/ml in 5% (w/v) TCA was used for estimating DNA concentrations in the samples.

2.7.2 Protein Estimation (Lowry).

Protein concentration was estimated using a modification of the method of Lowry et al, 1951. To ensure solubilisation of the

samples, determinations were carried out in the presence of SDS. Two stock solutions were used:-

Solution A	0.50% (w/v) $CuSO_4$
Solution B	0.40% (w/v) NaOH
	2.00% (w/v) Na_2CO_3
	0.02% (w/v) Na Tartarate
Folins Solution	1 part Solution A
	49 parts Solution B

Folins-Ciocalteau : 1:1 dilution with distilled water

Samples were diluted appropriately in PBS, 100μl of sample mixed with 2% (w/v) SDS (100μl), and to this was added Folins Solution (1ml). After 20 min freshly prepared Folins-Ciocalteau reagent (100μl) was added, with vortexing. After a further 20 min, absorbance was read at 750nm.

Standard curves were set up by the same procedure using bovine serum albumin in the range 100 - 600 μg/ml and PBS containing 1% (w/v) SDS as a blank.

2.7.3 Protein Estimation by A_{280nm}/A_{260nm} Measurement.

For rapid estimation of protein concentration, the method of Warburg and Christian, 1941, was employed. The absorbance of samples was measured at 280nm and 260nm, and the following formula used to calculate protein concentration:-

$$A_{280nm} \times f \times 1/d = \text{protein concentration (mg/ml)}$$

where d is the light path in cm and f is obtained from a table (Dawson et al, 1974) using the ratio A_{280nm}/A_{260nm}.

2.8 LIQUID CHROMATOGRAPHY.

2.8.1 Anion-exchange Chromatography of Proteins.

Small and large scale separation of proteins was undertaken using a 1ml Mono-Q column (Pharmacia) and a 300ml Q-Sepharose column (Pharmacia). All elution buffers in this section were made up in Milli-Q pure water and filtered through 0.22μm Durapore filters (Waters Associates) before use.

2.8.1.2 Small Scale Separation.

A Pharmacia Fast Protein Liquid Chromatography (FPLC) system with a 1ml Pharmacia Mono-Q column was utilised for small scale anion-exchange chromatography. Protein elution was monitored at 280nm with a Pharmacia UV-M monitor. Prior to sample application, the column was equilibrated with Buffer A (5mM Tris-HCl pH7.4, 2mM EDTA). Samples (20mg) were dialysed for 1h at 4^{o}C against two changes (21) of Buffer A containig 2mM DTT, before application. Elution was effected at 0.5ml/min with a linear gradient as follows :-

Time (min)	%A	%B (Buffer A + 1M NaCl)
Initial	100	0
4	100	0
34	50	50
40	0	100
50	100	0

Fractions (0.5 or 1 ml) were collected and kept on ice.

2.8.1.2 Large Scale Separation.

A 300ml column, packed with Q-Sepharose Fast-Flow (Pharmacia) was used for large scale separations. This was equilibrated at 4°C with 50mM Tris pH 7.4 at 4°C, 5mM EDTA. Sample (100ml, ~3g protein) was applied to the column and the column washed with 300ml equilibration buffer. Bound protein was eluted with a 1l linear gradient (formed by a stirred gradient former) of 0 - 0.5M NaCl in equilibration buffer at a flow rate of 1ml/min. Fractions (12ml) were collected and monitored for conductivity and absorbance at 280nm. All procedures were carried out at 4°C. An LKB Microperpex S peristaltic pump was utilised for maintaining buffer flow.

2.8.2 Gel Filtration Chromatography of Proteins.

Small and large scale separation of proteins was undertaken using a 26.5ml LKB UltroPac column and a 100ml Ultragel (Pharmacia) column. All elution buffers in this section were made up in Milli-Q pure water and filtered through 0.22μm Durapore filters (Waters Associates) before use.

2.8.2.1 Small Scale Separation.

An LKB UltroPac TSK-G 3000 SW gel permeation column (7.5 x 600 mm) was used to separate proteins according to their molecular size. Isocratic elution (0.5ml/min) was effected with pump A of a Pharmacia FPLC system. Elution buffer consisted of 0.1M sodium

sulphate, 1mM EGTA, 50mM HEPES pH7.4, 5% (v/v) glycerol. The column was equilibrated with this buffer prior to use. Samples (200µl, >1mg) were loaded and fractions (0.25ml) were collected. Protein was monitored at 280nm using a Pharmacia UV-M monitor.

2.8.2.2 Large Scale Separation.

A 1.5 x 100 cm Ultragel (Pharmacia) column, pre-equilibrated with 10mM Tris-acetate pH6, 1mM EDTA, 0.16M KCl was used for large scale gel filtration of proteins. Samples (1 - 2 ml, ~30mg) were loaded and eluted isocratically with 10mM Tris-acetate pH6, 1mM EDTA, 0.16M KCl, at 10ml/h, using an LKB Microperpex S pump. Fractions (2.5ml) were collected and monitored for protein at 280nm.

2.8.3 Immunoaffinity Chromatography.

2.8.3.1 Coupling of antibody to CNBr activated Sepharose.

Cyanogen bromide activated Sepharose (2g) was swollen at room temperature for 15min in 1mM HCl, washed with 400ml 1mM HCl using a glass sinter funnel, and the 'dry' Sepharose collected by centrifugation at 2000 rpm in a bench top centrifuge (MSE Centaur 1). The Sepharose was then resuspended in 0.1M $NaHCO_3$ pH9, 0.5M NaCl (10ml). Serum (rabbit anti-rlct) was then added to the gel (1ml serum per 3ml of gel), the pH was readjusted to 9 (when necessary) and the gel was rotated end over end at 4^oC over night. The Sepharose was collected by filtering on a glass sinter funnel and washed with 0.25M

$NaHCO_3$ pH9 until no protein was present in the eluent. To block unbound sites on the Sepharose, 20ml 1M ethanolamine-HCl pH9 was added and the gel was rotated end over end for 2h at room temperature. The gel was then washed with 50ml 0.5M $NaHCO_3$ pH 9, followed by 50ml 0.1M Borate + 1M NaCl pH 4.1 and finally with 50ml double distilled water. Columns were stored at 4^oC in 0.02% (w/v) sodium azide and washed thoroughly with distilled water before use.

2.8.3.2 Protein separation by immunoaffinity.

Cyanogen bromide activated Sepharose coupled to antibody was packed by gravity to give a 7ml (approx. 14 x 45 mm) column. Protein solution (>3mg) was run through this column, under gravity, the eluent collected and reapplied to the column. Unbound protein was washed off the column with 5 column volumes of PBS (or until the eluent was free of protein). The column was then washed with 0.25M Glycine-HCl pH2.5 (to remove weakly bound protein) until no protein was present in the eluent and the fractions collected (3.5ml) were neutralised immediatly with 20µl 2M Tris. To remove tightly bound protein, the column was washed with 0.25M Glycine HCl pH2.5 + 10% Dioxane until the eluent contained no protein. Again, fractions (3.5ml) were neutralised immediatly with 20ul 2M Tris. Fractions containing protein were passed down a PD10 column (Pharmacia) to remove the dioxane and the protein was eluted with PBS.

2.8.4 GTP-affinity Chromatography.

GTP-agarose was packed, by gravity, to give a 3ml (approx. 12 x 26 mm) column and equilibrated with 100mM Tris-HCl pH 7.5, 1mM EDTA. Protein solution (>1mg) was run through the column and the collected eluent reapplied. Unbound protein was removed by washing with equilibration buffer until no protein was present in the eluent. Bound protein was then eluted with equilibration buffer containing 1M KCl.

2.8.5 Reverse Phase High Performance Liquid Chromatography (HPLC).

All solutions were made up with Milli-Q water in acid washed glassware and all buffers were filtered through 0.22μm Durapore filters (Waters Associates) before use.

This technique was used for the analysis of ϵ(γ-glutamyl) lysine.

The reverse phase HPLC of amino acids and dipeptide was performed using a modification of the method of Griffin and Wilson (1984). A Nova-Pak C18 column (Millipore / Waters) was used instead of the Zorbax C8 column described in the published method.

The HPLC systems used consisted of a model 6000 and a model 510 pump, controlled by a model 660 gradient programmer (Millipore / Waters) with a data aquisition system consisting of a Nelson Analytical 900 series interface box and a Walters IBM compatible personal computer using Nelson Analytical software, or a System Gold HPLC (Beckman).

Samples were derivatised, pre-column, with OPA reagent at room temperature for 1.5min (30sec with vortexing) and 20μl applied to

the column. A Perkin-Elmer LS-1 fluorimeter (excitation = 340nm, emission = 455nm) was used for the detection of fluorescent derivatives. The solutions used are detailed below :-

Buffer A	20mM potassium acetate pH5.6
Buffer B	Methanol (HPLC grade)

(buffers A and B contained 1% (v/v) tetrahydrofuran)

OPA reagent	100mg OPA
	5ml 0.4M potassium borate buffer pH10.4
	5ml methanol (HPLC grade)
Derivatisation mix	50μl OPA reagent
	5μl 2-mercaptoethanol
	20μl sample

Elution was effected at a constant flow rate of 1.5ml/min after equilibration of the column with buffer A for 15min. The gradient programme for elution is as follows :-

Time (min)	%A	%B
Initial	80	20
35	20	80
40	0	100
45	0	100
55	80	20

2.8.6 Amino Acid Analyser.

To confirm the levels of $\epsilon(\gamma$-glutamyl)lysine quantitated by reverse phase HPLC, random samples were analysed by ion-exchange, on a bed of Dionex DC-4A resin (converted to the lithium form by the method of Benson, 1976) supported in a glass lined column (3 x 250 mm) within a water jacket, using a modification of the method of Griffin and Wilson, 1984, and the software produced by Griffin et al, 1988.

Elution was effected with a Waters 501 pump (Millipore / Waters) at a flow rate of 0.2ml/min. A Waters M-45 pump (Millipore / Waters) was used to pump OPA reagent at a flow rate of 0.2ml/min to a post-column mixing manifold (Waters Associates) from where a 2min reaction time was allowed prior to fluorescence detection. The fluorimeter used was a Perkin-Elmer 1000M (excitation = 340nm, emission = 455nm) connected to a Nelson Analytical 900 series interface box. Data manipulation and storage was facilitated by a Walters IBM compatible personal computer using Nelson Analytical software. Buffer reservoirs were connected through a rheodyne pressure-regulated selection valve (operated by compressed air) to the column. A BBC Model B microcomputer was used to control the elution programme which was as follows :-

Buffer	pH	Time (min)	Temp.^{o}C
A	2.90	38	35
B	3.04	28	35
C	2.95	45	35
D	3.39	45	35
E	3.44	20	35
0.3M LiOH, 10mM EDTA	12.50	30	60

The buffer system used was the lithium citrate Picobuffer System IV (Pierce).

OPA reagent (1l) was made up, the day before use, as follows :-

50g Boric acid

44g Potassium hydroxide

3.5ml Brij-35

600mg OPA in 7.5ml methanol (HPLC grade)

5ml 2-mercaptoethanol

Elution buffers and OPA reagent were made up with Milli-Q pure water, filtered through 0.22μm Durapore filters (Millipore / Waters) before use and kept in air tight containers (a dark bottle for the OPA reagent) under a nitrogen pressure of 0.35bar.

2.9 STATISTICAL ANALYSIS.

2.9.1 Standard Error.

Standard errors were calculated from the formula :-

$$SE = \bar{x}/\sqrt{n}$$

where $\bar{x}$ is the sample mean and n the number of observations.

2.9.2 Students t-test.

A two-sample t-test was used to estimate the difference between two means. This analysis was carried out using the MINITAB System (Minitab Inc., Enterprise Drive, State College, PA16801, U.S.A.). The following formula was used to calculate t :-

$$t = \frac{(\bar{x}_1 - \bar{x}_2)}{\sqrt{s_1^2/n_1 + s_2^2/n_2}}$$

Where t is the value from a t-table corresponding to a percentage confidence for the relevant degree of freedom (df) as defined below ($\bar{x}_1$ and $\bar{x}_2$ are the sample means, s_1 and s_2 the standard deviations and n_1 and n_2 the number of observations in each group). This test does not assume that the two data groups have equal standard deviations.

$$df = \frac{((s_1^2/n_1) + (s_2^2/n_2))^2}{\frac{(s_1^2/n_1)^2}{(n_1 - 1)} + \frac{(s_2^2/n_2)^2}{(n_2 - 1)}}$$

Thus, where two groups of data are said to be significantly different, the probability of them being from one group is less than, or equal to, 0.05.

2.9.3 Coefficient of correlation.

The Pearson Product-moment coefficient (r) was employed as a correlation coefficient :-

$$r = \frac{\sum x.y}{n.s_x.s_y}$$

Where n is the number of scores, and x and y are the two groups of data under analysis. The nearer r is to 1, the greater the correlation, with positive or negative signs indicating the direction of correlation.

3: METASTATIC VARIANTS OF ESTABLISHED CELL LINES AS A MODEL FOR THE ASSESSMENT OF THE RELATIONSHIP BETWEEN TRANSGLUTAMINASE ACTIVITY AND METASTATIC POTENTIAL.

3.1 INTRODUCTION.

The alterations in transglutaminase activity that occur during carcinogenesis and tumour growth are now well established (refer to section 1.3.5), however, little is known about the importance of this enzyme in the metastatic event. Work in this laboratory has suggested that metastasis may be connected with a further reduction in transglutaminase activity (Barnes et al, 1984, 1985; Hand et al, 1987). Workers in France have demonstrated reduced levels of activity in metastasizing rat rhabdomyosarcoma cells compared to their non-metastasizing counterparts (Delcros et al, 1986), and in malignant human intestinal cancer compared to benign, which in turn is lower than in the normal tissue (Delcros et al, 1987). This chapter details experiments, on a series of metastatic variants, aimed at establishing the importance of transglutaminase activity in metastasis.

3.1.1 Description of metastatic variants of an HSV-2 induced hamster fibrosarcoma.

A spontaneously metastasizing hamster fibroblast cell line (HSV-2-333-2-26) was originally obtained by in vitro transformation of hamster embryo fibroblasts with inactivated herpes simplex virus-2 (HSV-2). Following resection of the parent (HSV-2-333-2-26) tumour, individual lung metastases were removed, passaged in vivo, and established as in vitro cultures. The metastatic potential of these sublines and the parent line, was assessed as the number of animals who developed metastatic nodules out of the total number of animals whose

primary tumour load was resected (Teale and Rees, 1987) (Table 3.1).

As a control, the BHK-21 cell line was purchased from Flow Laboratories. These cells are 'normal' hamster embryo fibroblasts, from the kidney, and are susceptible to HSV-2 transformation.

3.1.2 Assessment, 'in vitro', of the invasive capacity of the variant cell lines.

To assess the invasive capabilities of cells in culture, a modification of the method of Repesh (1989) was used (section 2.2.4), whereby cells must adhere to, and pass through, a collagen based matrix in order to enter the lower chamber. The invasive capacity (Table 3.2) is given as the percentage of seeded cells that have entered the lower chamber. In all but one of the cell lines (Met C) the metastatic potential of the solid tumour is reflected by the invasive capacity of the cells in culture. However, the ability of a cell to invade does not automatically mean that that cell is capable of forming a metastatic growth. Many factors of the host environment must also be taken into consideration.

Table 3.1 : Incidence and location of secondary tumours after resection of the primary tumour load.

Cell line	Number with metastasis / number innoculated.	Lungs	Kidney	Regional lymph nodes.
Met D	1 / 20	0	0	1 / 20
Met C	4 / 18	0	0	4 /18
Parent	6 / 19	2 / 19	0	6 / 19
Met B	20 / 20	19 / 20	8 / 20	11 / 20
Met E	19 / 19	19 / 19	9 / 19	11 / 19
Met F	20 / 20	18 / 20	4 / 20	9 / 20

Primary subcutaneous tumours were resected and the animals observed for up to 12 weeks. Metastatic involvement was monitored for the lungs, kidneys and regional lymph nodes.

From Teale and Rees, 1987.

Table 3.2 : The invasive capacity of cultured hamster fibroblast variants.

Cell line	Metastatic potential 'in vivo'	Invasive capacity 'in vitro'
BHK	N/A	0
Met D	1/20	5.0±0.56
Met C	4/20	19.2±2.71
Parent	6/19	12.4±3.83
Met B	20/20	33.5±2.02
Met E	19/19	28.9±5.64
Met F	20/20	38.6±4.07

Invasive capacity is expressed as the percentage of cells able to traverse a collagen based matrix in a 72h period. 2×10^3 cells were seeded onto the upper surface of a Cellagen TM CD24 disc. Cell numbers on either side of the membrane were counted after incubation at $37^{o}C$ for 72h (section 2.2.4).

Data represent the mean of 3 experiments ± SE.

3.2 TRANSGLUTAMINASE ACTIVITY IN CULTURED CELLS.

Cultured cells, maintained in serum free medium for 15 h prior to experimentation, were homogenized and assayed for total transglutaminase activity. Homogenates were then subjected to non-ionic detergent extraction (section 2.4.2) and the two forms of tissue transglutaminase separated by anion-exchange chromatography (section 2.8.1.1). Fractions eluted from the anion-exchange column were assayed for transglutaminase activity with the ^{3}H-putrescine assay (section 2.6.1.2) to give activity profiles. Active fractions were then combined to give a particulate transglutaminase pool and a cytosolic transglutaminase pool. These pools were then assayed for activity by the ^{14}C-putrescine assay (section 2.6.1.1).

3.2.1 Measurement of total transglutaminase activity.

Total transglutaminase activity was measured in the parent cell line, five of its metastatic variants Met B, Met C, Met D, Met E and Met F and the normal BHK cell line (Table 3.3). Values are expressed as functions of DNA or protein to enable comparison of transglutaminase levels in vitro and in vivo (section 3.3).

Reductions in transglutaminase activity were observed in the sequence BHK > Met D > Met C > parent and Met F > Met B and Met E (Fig.3.1). Statistical analysis of the mean values showed that whatever function was used to express the data, activity in the normal BHK cells was significantly greater than activity in the weakly metastatic cell lines, Met D, C and parent, which in turn was

Table 3.3 : Transglutaminase activity in cultured cell sub-lines, of an HSV-2 induced hamster fibrosarcoma, of differing metastatic potential.

Cell Line	Metastatic potential.	Transglutaminase Activity.		
		10^6 cells	U/mg DNA	U/mg protein
BHK		9.3±0.5	1206±107	21.3±1.17
Met D	1/20	5.1±0.2	740±92	10.7±0.41
Met C	4/20	4.8±0.2	502±55	9.9±0.86
Parent	6/19	4.6±0.4	447±18	9.6±0.30
Met B	20/20	1.3±0.1	182±57	5.6±0.96
Met E	19/19	1.9±0.4	211±36	5.9±0.70
Met F	20/20	3.9±0.3	500±78	9.1±0.50

Cells were harvested, homogenised (10^7 cells in 300ul of homogenising buffer) and assayed for transglutaminase activity according to the procedures outlined in sections 2.2.1; 2.4.1; 2.6.1.1 respectively.

Data represents mean values of 4 separate experiments ± SE.

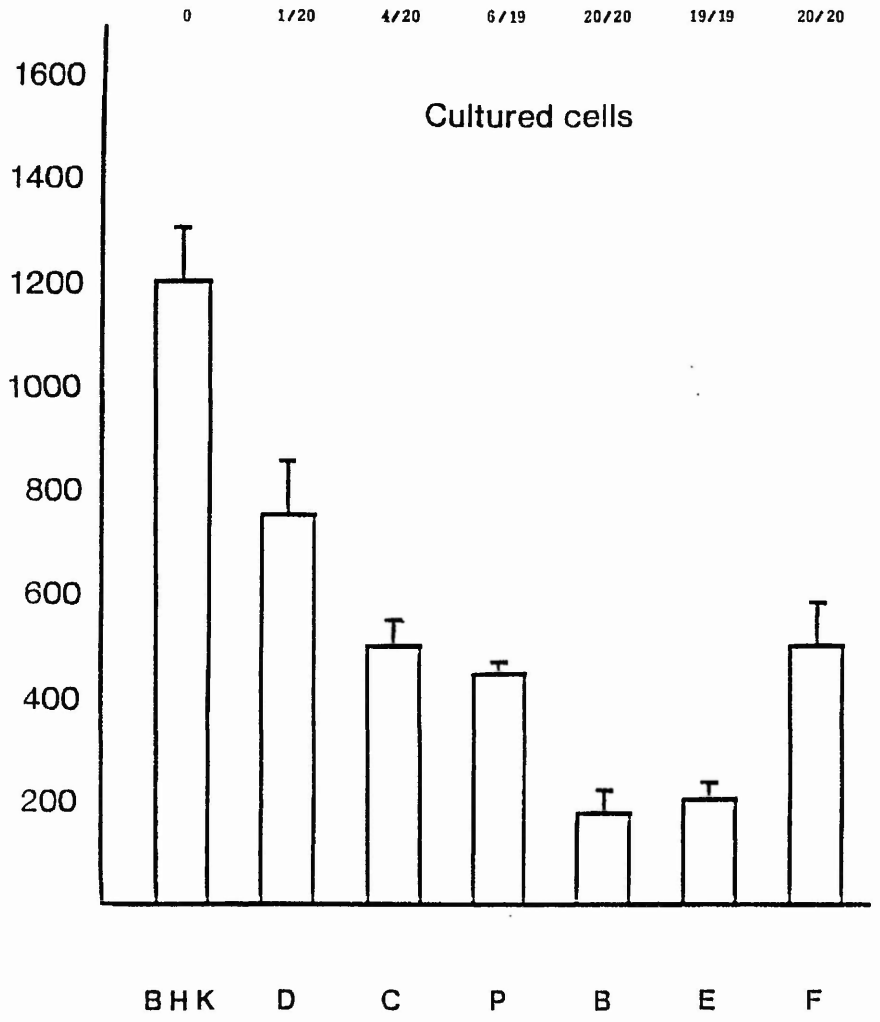
Metastatic potential is defined as the number of animals with metastatic involvement out of the number of animals whose primary load had been resected.

Fig. 3.1 : Total transglutaminase activity in cultured cells.

Cells were harvested by incubating for 5min in 5mM Tris, 2mM EDTA (TE), pelleted gently in an MSE Centaur bench top centrifuge (1500 rpm), washed twice with TE, resuspended in 600ul (10^7 cells per 300ul) of cold homogenising buffer and then homogenised (section 2.4.1). Homogenates (~20mg protein / ml) were assayed for transglutaminase by the Ca^{2+} dependent incorporation of ^{14}C-putrescine into N,N'dimethylcasein (section 2.6.1.1).

Metastatic potential is shown above each column.

Data represents the mean of 6 experiments ± SE.


0
1/20
4/20
6/19
20/20
19/19
20/20
Cultured cells
1600
1400
1200
1000
800
600
400
200
BHK
D
C
P
B
E
F

significantly greater than that in the highly metastatic lines Met B and E. The level of activity in the highly metastatic Met F cell line was significantly different to that found in the other highly metastatic lines, but instead was comparable with that found in the weakly metastatic lines. The coefficient of correlation (section 2.9.3) for metastatic potential and transglutaminase activity is r = -0.835, suggesting that an inverse relationship may exist between these two parameters.

3.2.2 Distribution of transglutaminase activity.

Since tissue transglutaminase has been shown to exist in two separate forms within normal rat liver (Chang and Chung, 1986) and that following the chemical induction of hepatocellular carcinomas it is the activity of the cytosolic form which is considerably decreased in these tumours (Hand et al, 1988), it was necessary to establish, firstly, whether two forms of the enzyme existed in these cell lines, and secondly, if any alterations to their respective levels could be demonstrated.

Total transglutaminase activity was therefore extracted with non-ionic detergent. This detergent extract was then separated on a Mono-Q anion-exchange column with a 0 - 0.5 M NaCl gradient as the eluent. Eluent fractions, when assayed for transglutaminase activity, indicated the presence of two distinct activity peaks, the first eluting at 0.15 - 0.25 M NaCl and the second at 0.35 - 0.45 M NaCl (Fig.3.2a). This elution profile was similar to that established previously for rat liver (Chang and Chung, 1986; Hand et al, 1988) when it was demonstrated that the activity in the first peak was due to membrane

Fig. 3.2 : Typical anion-exchange chromatography elution profiles of transglutaminase activity from cultured cells.

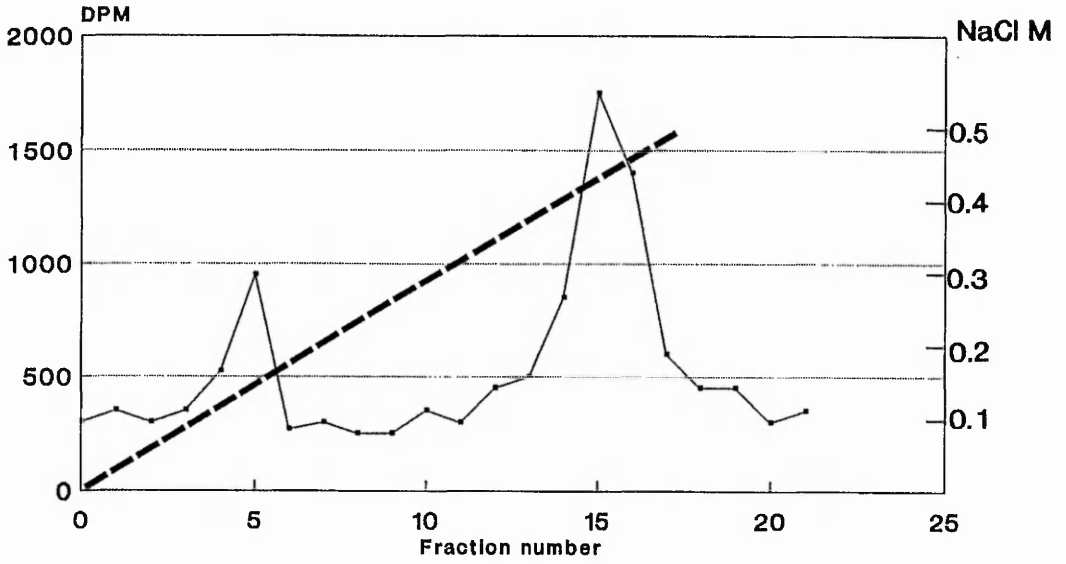
Cells were harvested by incubating for 5min in 5mM Tris, 2mM EDTA (TE), pelleted gently in an MSE Centaur 1 bench top centrifuge (1500 rpm), washed twice with TE, resuspended in 600ul (10^7 cells per 300ul) of cold homogenising buffer and then homogenised (section 2.4.1).

a) Cells were homogenised in buffer and separated into a 71,000g pellet and a cytosol fraction by centrifugation. The pellet was then extracted with non-ionic detergent by resuspension of the pellet in buffer containing 1% (w/v) Lubrol-PX and being left on ice for 1h before being centrifuged to give a particle free supernatant (PFS) and a 71,000g pellet (section 2.4.2). Proteins (~5mg) in the PFS were separated by anion-exchange chromatography on a Pharmacia Mono-Q column (1ml) using a 0 - 0.5 M NaCl gradient (section 2.8.1.1). Eluent fractions, 500ul, were assayed for the presence of transglutaminase activity by the ^{3}H-putrescine assay (section 2.6.1.2).

b) Cells were homogenised in buffer (without lubrol) and separated into a cytosolic fraction and a 71,000g pellet by centrifugation (section 2.4.3). Proteins in the cytosolic fraction were separated by anion-exchange as above. The 71,000g pellet was resuspended in a volume of homogenising buffer, containing 1% (w/v) Lubrol-PX, equal to that of the cytosol, left on ice for 1h and separated into PFS and 71,000g pellet by centrifugation (section 2.4.2). Proteins present in the PFS were then separated by anion-exchange chromatography as above. Eluent fractions, 500ul, from the cytosol and from the PFS, were assayed for the presence of transglutaminase activity by the ^{3}H-putrescine assay (section 2.6.1.2).

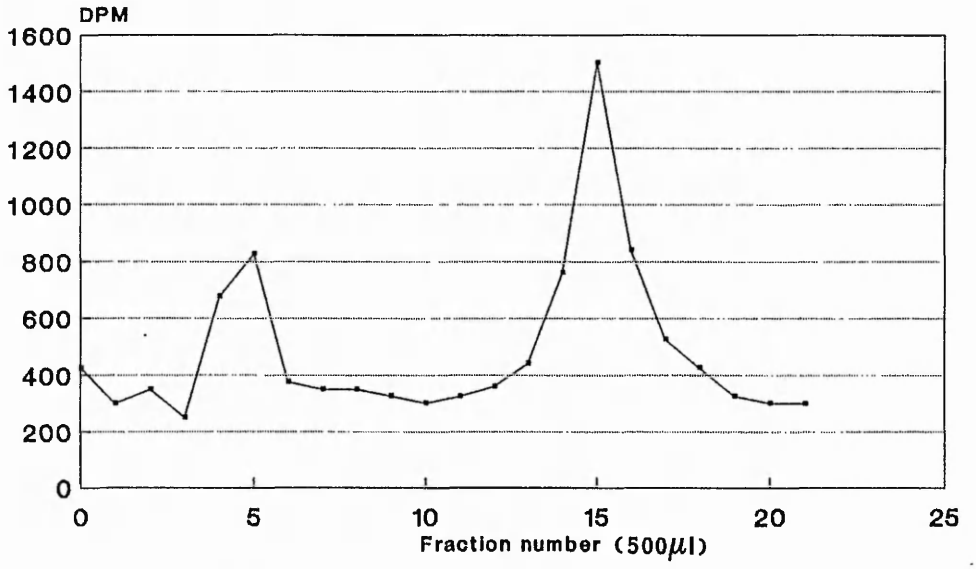
c) A_{280}nm trace of cellular proteins separated as in (a) above.

Figure 3.2a



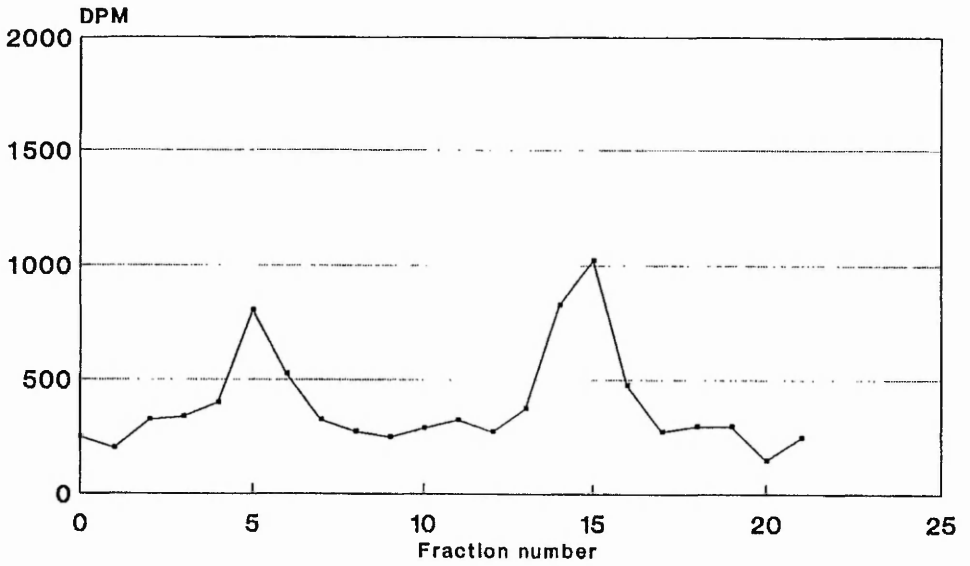
BHK

Figure 3.2a



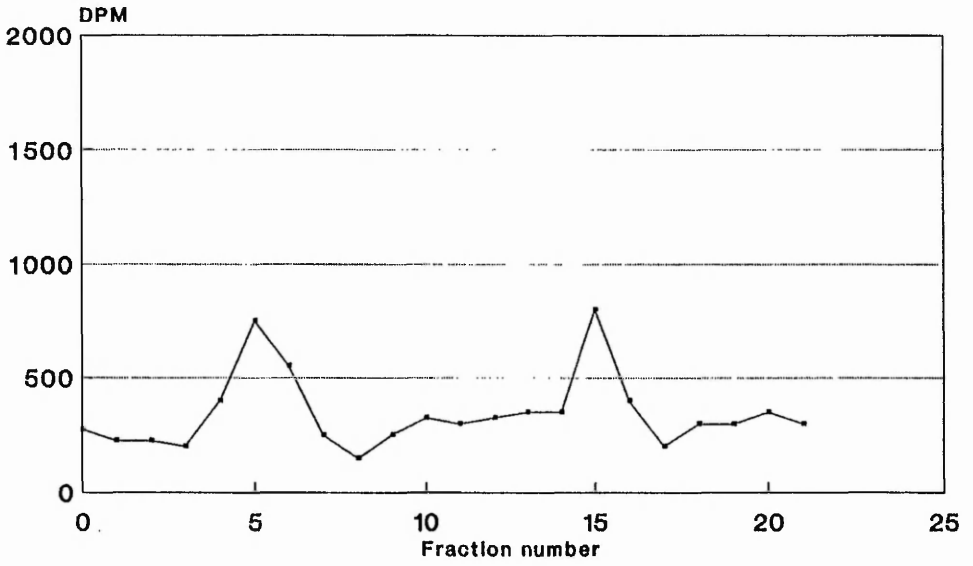
Met D

Figure 3.2a



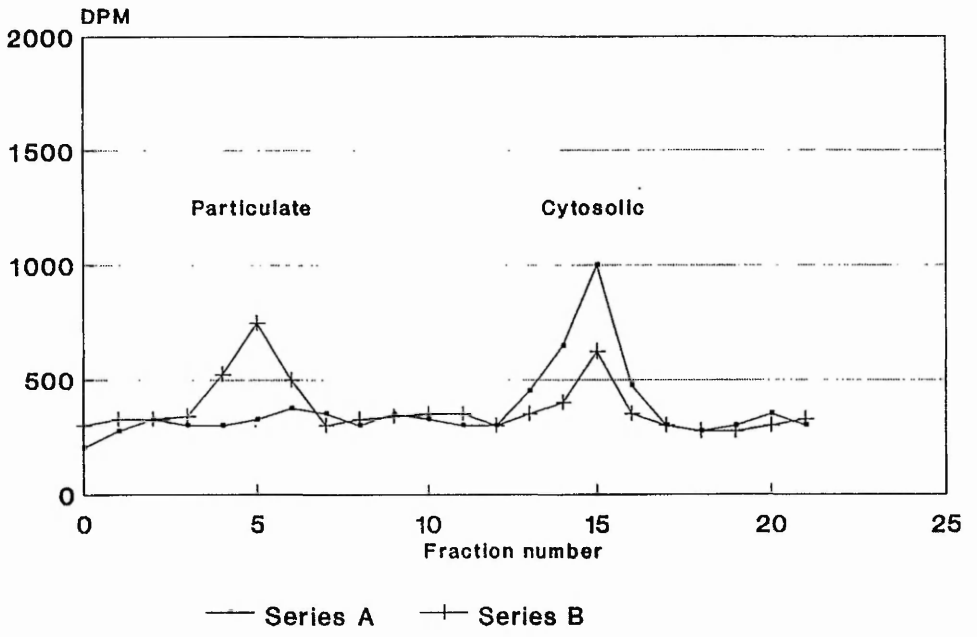
Parent

Figure 3.2a



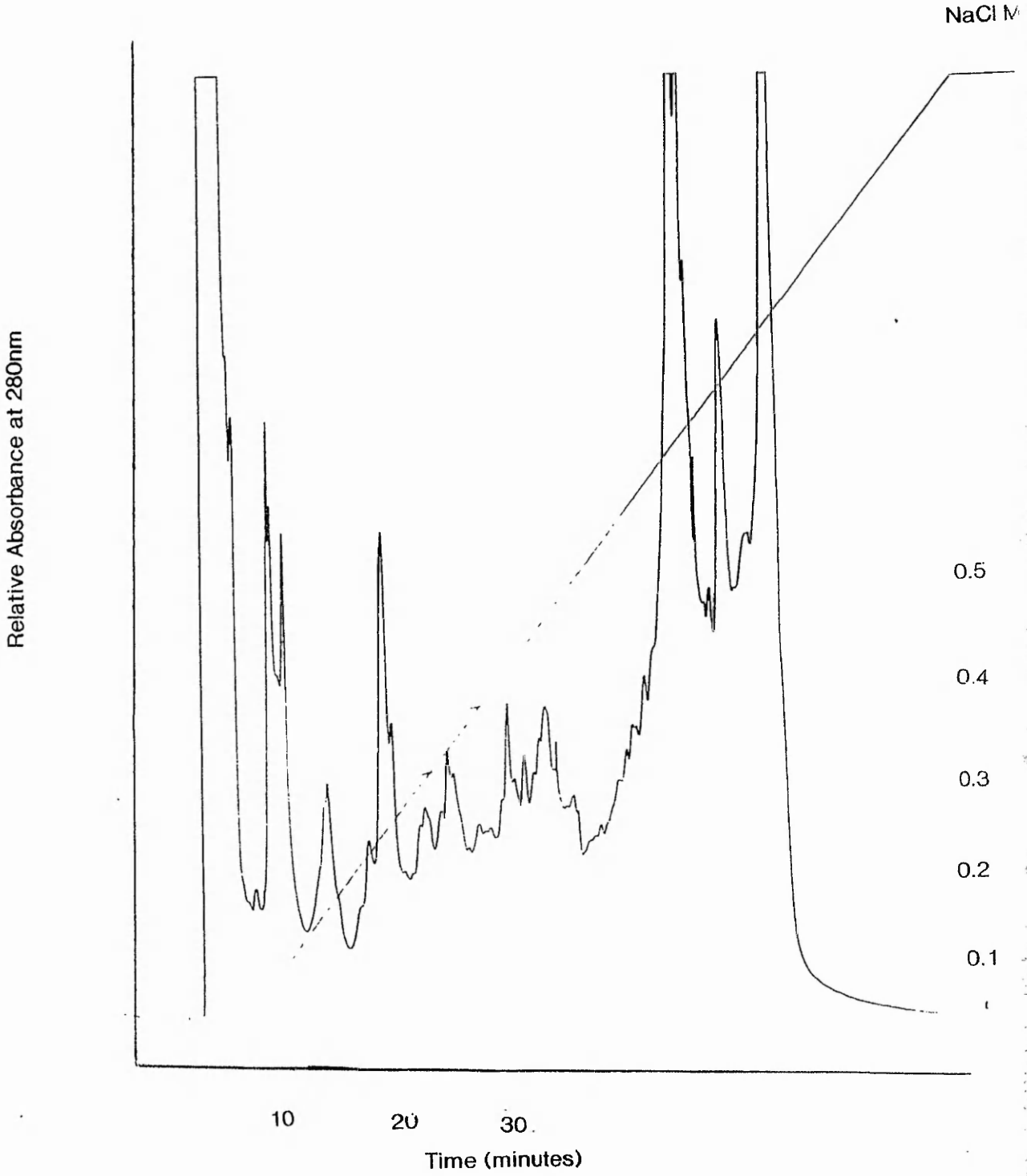
Met B

Figure 3.2b



Parent

Detergent extract


NaCl M
Relative Absorbance at 280nm
0.5
0.4
0.3
0.2
0.1
10
20
30
Time (minutes)

associated transglutaminase and that present in the second peak due to cytosolic enzyme. To confirm these results, a comparison was made between the elution profiles of cell homogenate particle-free supernatant pre- and post- non-ionic detergent extraction (Fig.3.2b). Only the second peak of activity (0.35 - 0.45 M NaCl) was detected pre-extraction, whilst the first activity peak (0.15 - 0.25 M NaCl) and a reduced second peak of activity were detected in the detergent extract. It was therefore confirmed that the membrane associated enzyme form gave rise to peak 1, and that peak 2 was due to the soluble cytosolic enzyme form. Measurement of transglutaminase distribution and activity is shown in Table 3.4. Particulate transglutaminase activity did not differ significantly between the seven cell lines, whether expressed as a function of cell number, DNA or protein. The levels of cytosolic transglutaminase activity, however, reflect the differences of total transglutaminase levels seen between cell lines. The coefficient of correlation for total transglutaminase activity and cytosolic transglutaminase activity being $r = 0.936$, whilst that for total transglutaminase activity and particulate activity is $r = -0.26$. Thus the differences in total transglutaminase activity, seen between cell lines, appear to be due specifically to alterations in the level of cytosolic transglutaminase activity.

Table 3.4 : Cytosolic and particulate transglutaminase activity in cultured cell sub-lines of an HSV-2 induced hamster fibrosarcoma of differing metastatic potential.

Enzyme activity was extracted from cell homogenates and separated as detailed in the legend to Fig.3.2a. Transglutaminase activity in the pooled active fractions was quantitated as in section 2.6.1.1. The metastatic potential of each cell line is given in parentheses.

Data represents the mean of 4 experiments ± SE.

Cell line		Transglutaminase activity.		
		$U/10^6$ cells	U/mg DNA	U/mg protein
BHK (0)	Cytosolic	6.7±0.34	913.8±53	18.27±0.23
	Particulate	1.52±0.10	246.2±29	4.11±0.37
	Cytosolic:Particulate	4.41 : 1	3.71 : 1	4.44 : 1
Met D (1/20)	Cytosolic	3.3±0.27	491.7±16	8.51±0.11
	Particulate	1.45±0.05	216.1±27	3.74±0.23
	Cytosolic:Particulate	2.27 : 1	2.28 : 1	2.27 : 1
Met C (4/20)	Cytosolic	3.1±0.19	449.5±37	7.60±0.33
	Particulate	1.5±0.05	217.5±41	3.67±0.20
	Cytosolic:Particulate	2.06 : 1	2.07 : 1	2.07 : 1
Parent (6/19)	Cytosolic	2.6±0.25	403.4±24	6.2±0.32
	Particulate	1.4±0.05	217.1±19	3.4±0.14
	Cytosolic:Particulate	1.8 : 1	1.85 : 1	1.82 : 1
Met B (20/20)	Cytosolic	1.0±0.3	162.1±17	2.61±0.12
	Particulate	1.4±0.04	226.9±28	3.65±0.31
	Cytosolic:Particulate	0.72 : 1	0.71 : 1	0.71 : 1
Met E (19/19)	Cytosolic	1.3±0.21	209.3±24	3.12±0.27
	Particulate	1.6±0.04	265.6±38	3.96±0.29
	Cytosolic:Particulate	0.81 : 1	0.78 : 1	0.79 : 1
Met F (20/20)	Cytosolic	2.9±0.22	475.3±36	7.04±0.31
	Particulate	1.4±0.03	229.4±33	3.40±0.15
	Cytosolic:Particulate	2.07 : 1	2.07 : 1	2.07 : 1

3.3 TRANSGLUTAMINASE ACTIVITY DURING GROWTH OF HAMSTER FIBROSARCOMAS.

Since a host animal may exert various selective environmental pressures on a growing tumour, influencing both enzyme activity and the ability of that tumour to invade and colonise new locations, it became evident that transglutaminase activity should be measured not only in cultured cell lines, but also in the solid tumours derived from these cell lines.

Homogenates of non-necrotic tumour tissue, removed at 20, 24, 28 and 34 days post-innoculation, were assayed for total transglutaminase activity and then extracted with non-ionic detergent prior to anion-exchange chromatography to assess the distribution of enzyme activity.

3.3.1 Measurement of total transglutaminase activity in 20 day old tumours.

Total transglutaminase activity was measured in 20 day old tumours derived from the parent cell line and four of its metastatic variants, Met B, Met D, Met E and Met F (Table 3.5). The data shown are mean values of 3 tumours, 2 measurements per tumour, expressed as functions of wet weight tissue, DNA or protein.

Activity, expressed as a function of DNA or protein, in tumours derived from the parent, Met D, Met B and Met E cell lines, was significantly higher than the levels observed in cultured cells; activity in Met F tumours was comparable to the higher than expected

levels found in cultured Met F cells. The trend of decreasing transglutaminase activity with increasing metastatic potential, seen in vitro, was confirmed in vivo (Met D > parent > Met F, Met E and Met B), with the aberrent Met F line conforming to the inverse relationship when under the influence of the host environment (Fig.3.3). The coefficient of correlation between metastatic potential and transglutaminase activity is r = -1.00, an improvement on that found in vitro.

3.3.2 Measurement of total transglutaminase activity during tumour growth.

Total transglutaminase activity was measured during the growth of the following tumour lines:- parent, Met B, Met D, Met E and Met F (Table 3.5), and is expressed as functions of wet weight tissue, DNA or protein.

Transglutaminase activity in the tumours derived from the parent cell line remained steady with no significant overall alteration occurring during tumour growth. However, there was a wide variation between the levels of activity found in individual tumours. Tumours derived from the highly metastatic variants, Met B, E and F, had initial (20 days after tumour induction) transglutaminase activities approximatly 40% lower than the less metastatic parent and Met D tumours. Furthermore, during the growth of these highly metastatic tumours, transglutaminase activity continued to decline resulting in a significant loss (approximately 50%) in the measurable activity present in these tumours at 34 days compared to that present at 20 days (Fig.3.4).

Table 3.5 : Total transglutaminase activity during the growth of solid tumours derived from metastatic variants of a HSV-2 induced hamster fibrosarcoma.

Tumours (200 - 500 mg) were harvested at the days indicated and homogenised to give a 20% (w/v) homogenate, as in section 2.4.1. Transglutaminase activity in the homogenate was measured as in section 2.6.1.1. Metastatic potential is shown in parentheses.

Data represent the mean values ± SE of three separate experiments.

Variant	Tumour age / days	Transglutaminase activity. U/g tissue	U/mg DNA	U/mg protein
Met D (1/20)	20	742.8±56.4	1528±153	8.79±1.86
	34	691.6±86.2	1181±211	8.21±0.75
Parent (6/19)	20	656.2±61.6	1160±82.6	8.55±1.66
	24	657.3±84.3	1220±197.2	7.46±0.66
	28	681.1±141	1162±69.4	7.19±1.1
	34	641.6±97	1064+79.3	7.49±1.3
Met B (20/20)	20	418.2±16.7	696.6±52.6	5.64±0.5
	24	422.3±15.5	703.6±22.5	4.12±0.2
	28	323.6±26.2	588.6±45	3.44±0.5
	34	215.6±20.5	405.7±27	2.38±0.3
Met E (19/19)	20	456.4±31.2	795.4±43	6.27±0.7
	34	229.1±28.0	387.7±38	2.03±0.4
Met F (20/20)	20	421.3±18.5	717.6±16.4	6.0±0.6
	24	392.3±11.4	625.8±4.1	3.72±0.3
	28	290.3±26.6	509.1±9.0	3.21±0.3
	34	199.0±12.6	368.7±49	2.86±0.5

Fig. 3.3 : Total transglutaminase activity in 20 day old hamster fibrosarcomas.

Non-necrotic tissue was removed from tumours 20 days after initiation of tumour growth. Tissue, 200mg, was homogenised as in section 2.4.1 to give a 20% (w/v) homogenate and this homogenate (~20mg protein / ml) then assayed for transglutaminase activity by the ^{14}C-putrescine assay (section 2.6.1.1).

Metastatic potential is shown above each column.

Data represents the mean of three experiments ± SE.

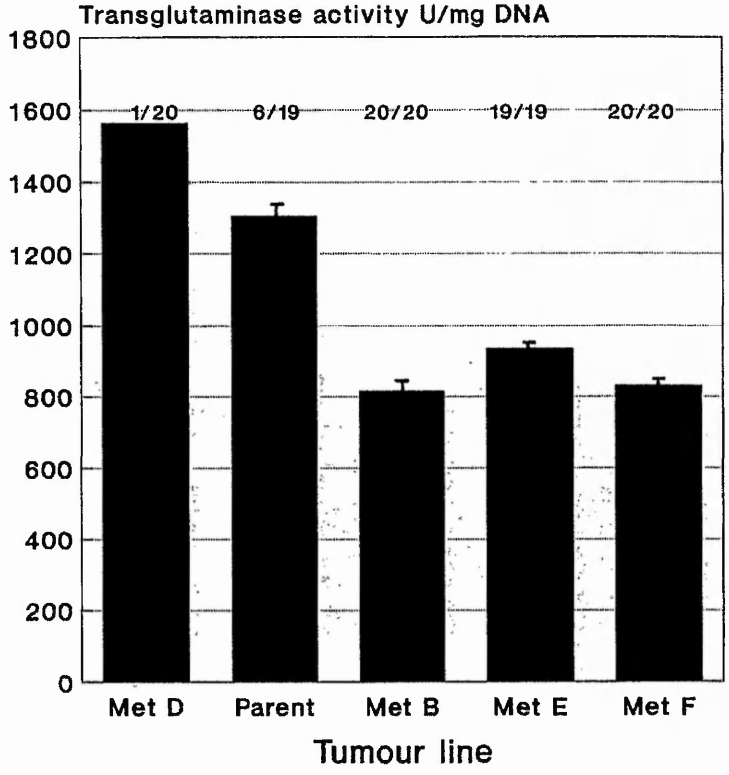
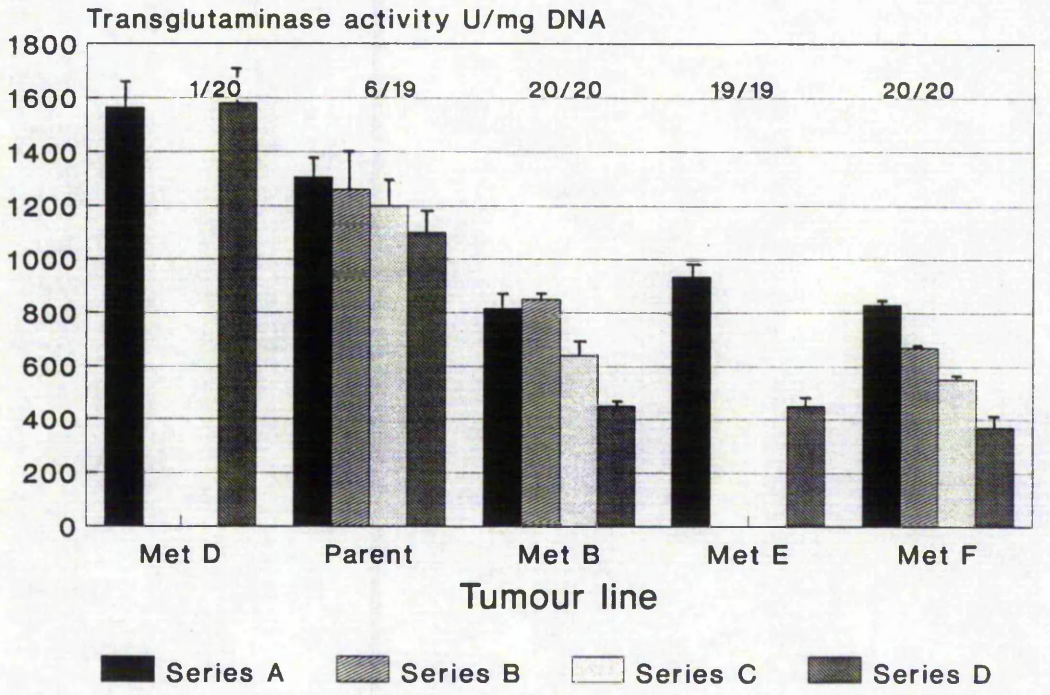

Transglutaminase activity U/mg DNA
1800
1600
1400
1200
1000
800
600
400
200
0
1/20
6/19
20/20
19/19
20/20
Met D
Parent
Met B
Met E
Met F
Tumour line

Fig. 3.4 : Total transglutaminase activity during the growth of hamster fibrosarcomas.

Non-necrotic tissue was removed from tumours at 20, 24, 28 and 34 days after initiation of tumour growth. Tissue, 200mg, was homogenised as in section 2.4.1 to give a 20% (w/v) homogenate and this homogenate (~20mg protein / ml) then assayed for transglutaminase activity by the ^{14}C-putrescine assay (section 2.6.1.1).

Metastatic potential is shown above each column.

Data represents the mean of three experiments ± SE.



A:d.20 B:d.24 C:d.28 D:d.34

3.3.3 Distribution of transglutaminase activity during tumour growth.

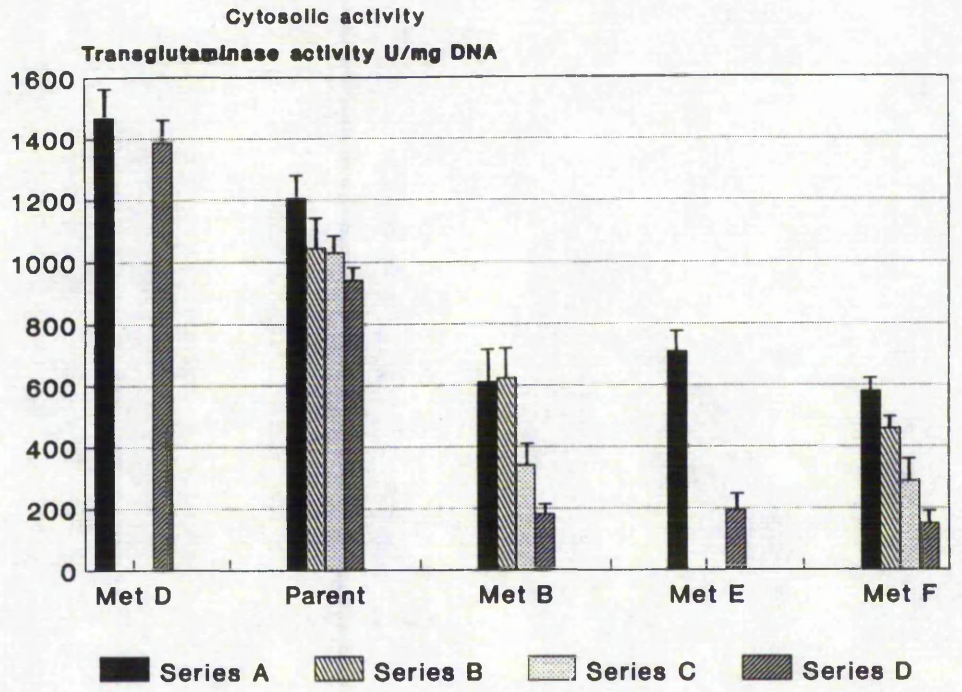
Eluent fractions, from anion-exchange chromatography of the particle-free supernatant from detergent extracted tumour homogenates. when assayed for transglutaminase activity, indicated the presence of the particulate and cytosolic forms of the enzyme.

It was found that the activity of the membrane associated form of the enzyme remained constant during the growth of tumours derived from the parent cell line and those tumours derived from its highly metastatic variants Met B, E and F (Fig.3.5). Interestingly, the amount of membrane associated activity present in the parent cell derived tumours was generally lower than that found in the tumours derived from the highly metastatic Met B and Met F lines, and in some parent tumours (3 out of 12) particulate activity was not detectable. However, the cytosolic activity present in the parent tumours was approximately twice that found in tumours from the two highly metastatic variants. During growth, the activity of the cytosolic enzyme found in the parent tumour did not alter significantly, although individual tumours showed a high degree of variation of cytosolic activity. In the highly metastatic variants a significant reduction in cytosolic enzyme activity corresponding to 70% occurred between 20 and 34 days, with individual activity levels falling within a small range (Fig.3.5). Thus, in the poorly metastatic parent tumours, the ratio of cytosolic activity to membrane associated activity fell from 8.5:1 to 7.5:1; the same ratio in the highly metastatic Met B and Met F tumours fell from 2.8:1 to 0.65:1 over the same time period (Table 3.6).

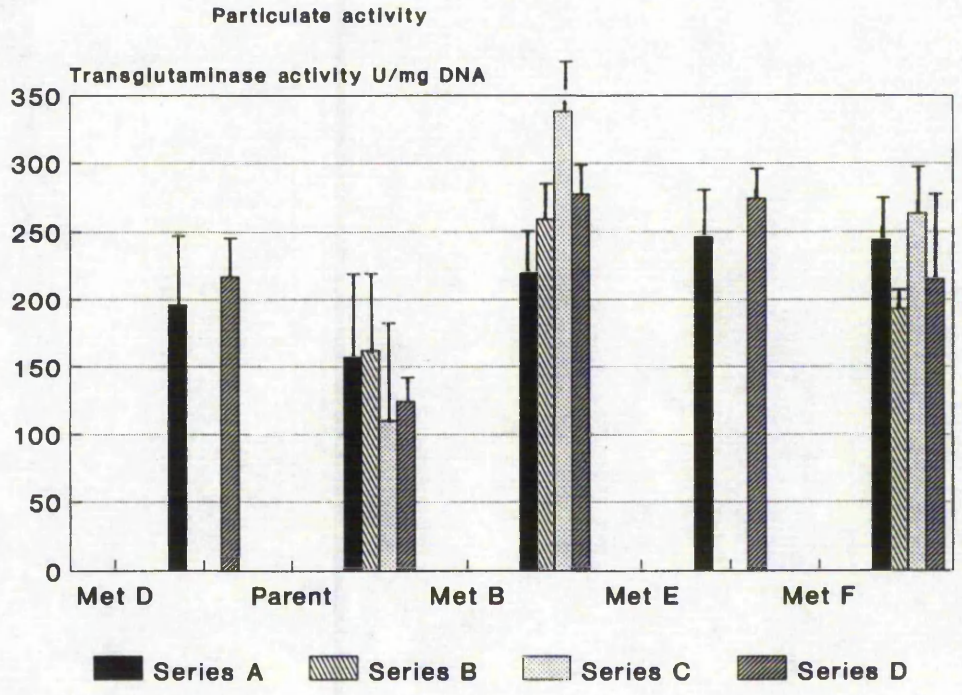
Fig. 3.5 : Particulate and cytosolic transglutaminase levels in hamster fibrosarcomas during their growth.

Non-necrotic tissue was removed from tumours 20 days after initiation of tumour growth. Tissue, 200mg, was homogenised as in section 2.4.1 to give a 20% (w/v) homogenate and separated into cytosolic and particulate fractions by centrifugation (section 2.4.3). The 71,000g pellet was then twice extracted with non-ionic detergent (Lubrol-PX), as in section 2.4.2, and the two resultant PFS's combined with the cytosolic fraction. After dialysis for 1h against two changes of 5mM Tris-HCl pH7.4, 2mM EDTA, 2mM DTT (21), the proteins in the combined supernatants were separated by anion-exchange chromatography (~5mg protein loaded) (section 2.8.1.1) and the fractions assayed for transglutaminase activity by the ^{3}H-putrescine assay (section 2.6.1.2). Activity in the pooled active fractions was then measured by the ^{14}C-putrescine assay given in section 2.6.1.1.

Data represents the mean of 3 experiments ± SE.



A:day 20, B:day 24, C:day 28, D:day34



A:day 20, B:day24, C:day 28, D:day 34

Table 3.6 : Levels of cytosolic and particulate transglutaminase activity in HSV-2 induced hamster fibrosarcomas during the growth of the solid tumour.

Tumours were harvested at the days shown. Non-necrotic tissue (200 - 500 mg) was homogenised to give a 20% (w/v) homogenate and the transglutaminase extracted as detailed in the legend to Fig.3.5. Activity was assayed as detailed in section 2.6.1.1.

The figures in parentheses show metastatic potential.

Data represents the mean values of six separate experiments ± SE.

Variant		Transglutaminase activity U/mg DNA			
		Day 20	Day 24	Day 28	Day 34
Met D (1/20)	Cytosolic	1468.3 ±108			1387.2 ±78
	Particulate	196.1 ±54			217.6 ±27
Cytosolic : Particulate		7.5:1			6.4:1
Parent (6/19)	Cytosolic	1204.6 ±75	1044.6 ±114	1030.2 ±54	941.3 ±34
	Particulate	157.3 ±67	162.7 ±59	110.2 ±79	124.3 ±13
Cytosolic : Particulate		7.6:1	6.4:1	9.3:1	7.6:1
Met B (20/20)	Cytosolic	612.63 ±115	623.6 ±103	339.6 ±73	181.3 ±22
	Particulate	220.3 ±40	259.1 ±24	338.7 ±43	277.4 ±19
Cytosolic : Particulate		2.8:1	2.4:1	1.0:1	0.65:1
Met E (19/19)	Cytosolic	709.4 ±65			193.5 ±36
	Particulate	246.5 ±31			274.3 ±21
Cytosolic : Particulate		2.9:1			0.7:1
Met F (20/20)	Cytosolic	580.2 ±26	458.5 ±21	287.3 ±75	150.8 ±25
	Particulate	244.6 ±21	193.8 ±8	263.7 ±35	215.3 ±71
Cytosolic : Particulate		2.4:1	2.3:1	1.1:1	0.7:1

Again, these results confirm the in vitro finding that differences in the level of total transglutaminase activity are due to specific alterations in the activity of the cytosolic form of the enzyme.

3.3.4 $\epsilon(\gamma$-glutamyl)lysine levels.

Since the protein crosslink, $\epsilon(\gamma$-glutamyl)lysine, is a major product of transglutaminase activity (Folk, 1972), alterations in enzyme activity should be reflected by alterations in the amount of $\epsilon(\gamma$-glutamyl)lysine present. Hence to reinforce the results of a direct in vitro assay for transglutaminase activity, the endogenous activity was monitored by following the levels of $\epsilon(\gamma$-glutamyl)lysine in tumour tissue. Tumour and cell homogenates were first digested by a range of proteolytic enzymes (section 2.6.2.1). The digested material was then passed down an ion-exchange column (section 2.6.2.2) to remove unwanted amino-acids, prior to analysis of the eluent fractions containing $\epsilon(\gamma$-glutamyl)lysine by reverse phase high performance liquid chromatography (section 2.8.5) (Fig.3.6a). Random samples were also assayed for $\epsilon(\gamma$-glutamyl)lysine content on an amino-acid analyser (section 2.8.6) (Fig.3.6c) to verify the results obtained by HPLC.

Tumours derived from the poorly metastatic parent cell line - harvested at twenty days after induction - contained approximatly double the amount of $\epsilon(\gamma$-glutamyl)lysine as those tumours derived from the two highly metastatic cell lines. Furthermore, the level in the parent tumours did not drop significantly between 20 and 34 days, whilst in the Met B and Met F tumours a significant decrease of approximatly 80% occurred during the same time interval (Fig.3.7).

These data therefore showed an excellent correlation ($r = 0.956$) to the reduction in activity of the cytosolic form of tissue transglutaminase found in the various tumours.

Fig. 3.6 : Chromatograms illustrating the separation of $\epsilon(\gamma$-glutamyl) lysine.

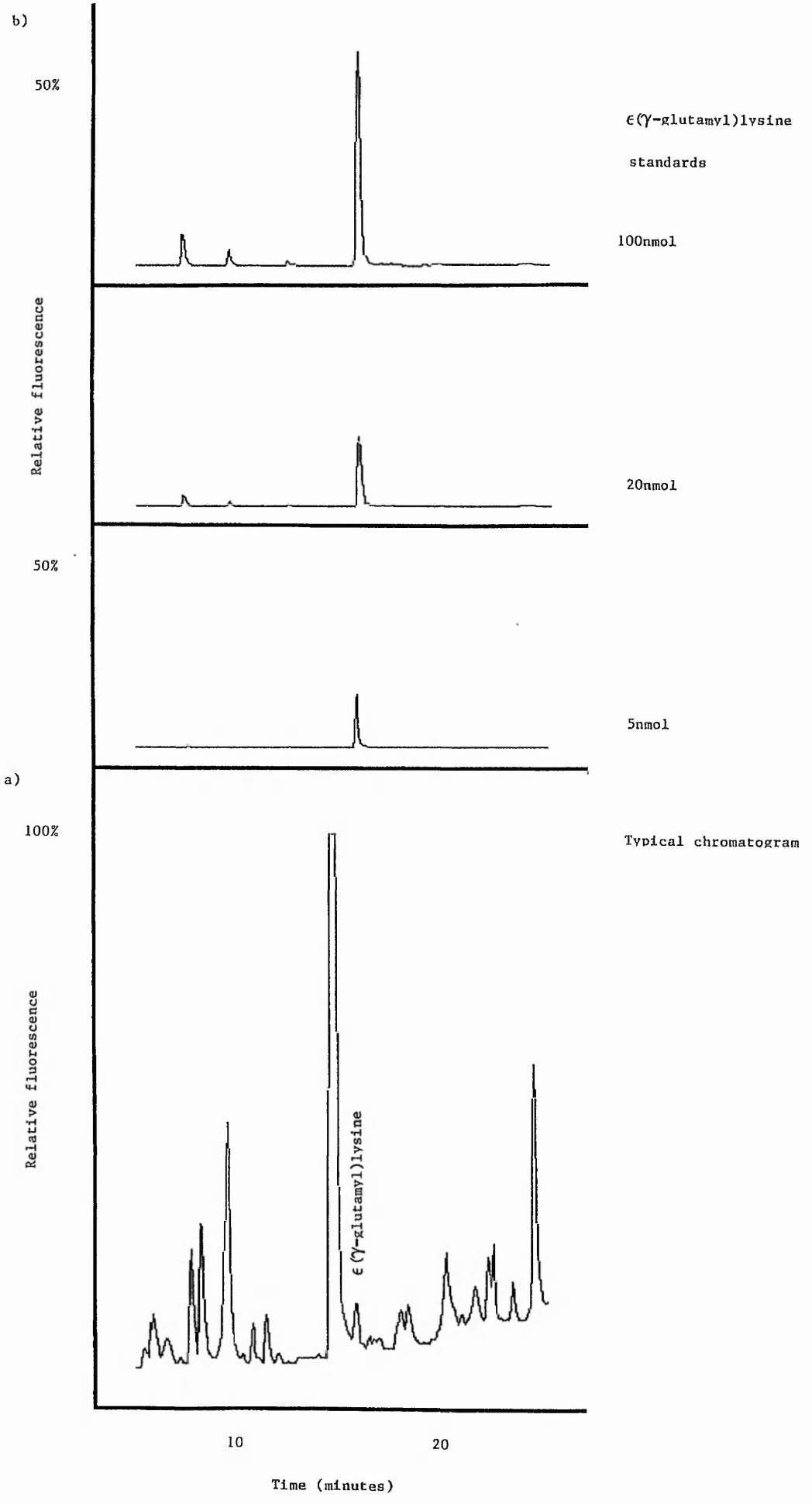
Homogenates (10mg protein) were digested enzymatically as in section 2.6.2.1 and prepurified by cation-exchange chromatography (section 2.6.2.2).

a) Chromatogram showing a typical separation of amino acids (~400 nmol) from dipeptide (~4nmol) by reverse phase HPLC on a NovaPak C_{18} column (section 2.8.5).

b) Reverse phase HPLC chromatogram showing the elution of $\epsilon(\gamma$-glutamyl) lysine standard (5nmol; 20nmol; 100nmol) from a NovaPak C_{18} column.

c) Chromatogram showing a typical separation of amino acids (~1umol) from dipeptide (~10nmol) by ion-exchange on an amino-acid analyser (section 2.8.6).

d) Chromatogram showing the elution of standard $\epsilon(\gamma$-glutamyl)lysine (20nmol) from the ion-exchange column of the amino acid analyser.


b)
50%
Relative fluorescence
ε(γ-glutamyl)lysine
standards
100nmol
20nmol
50%
5nmol
a)
100%
Relative fluorescence
Typical chromatogram
ε(γ-glutamyl)lysine
10
20
Time (minutes)

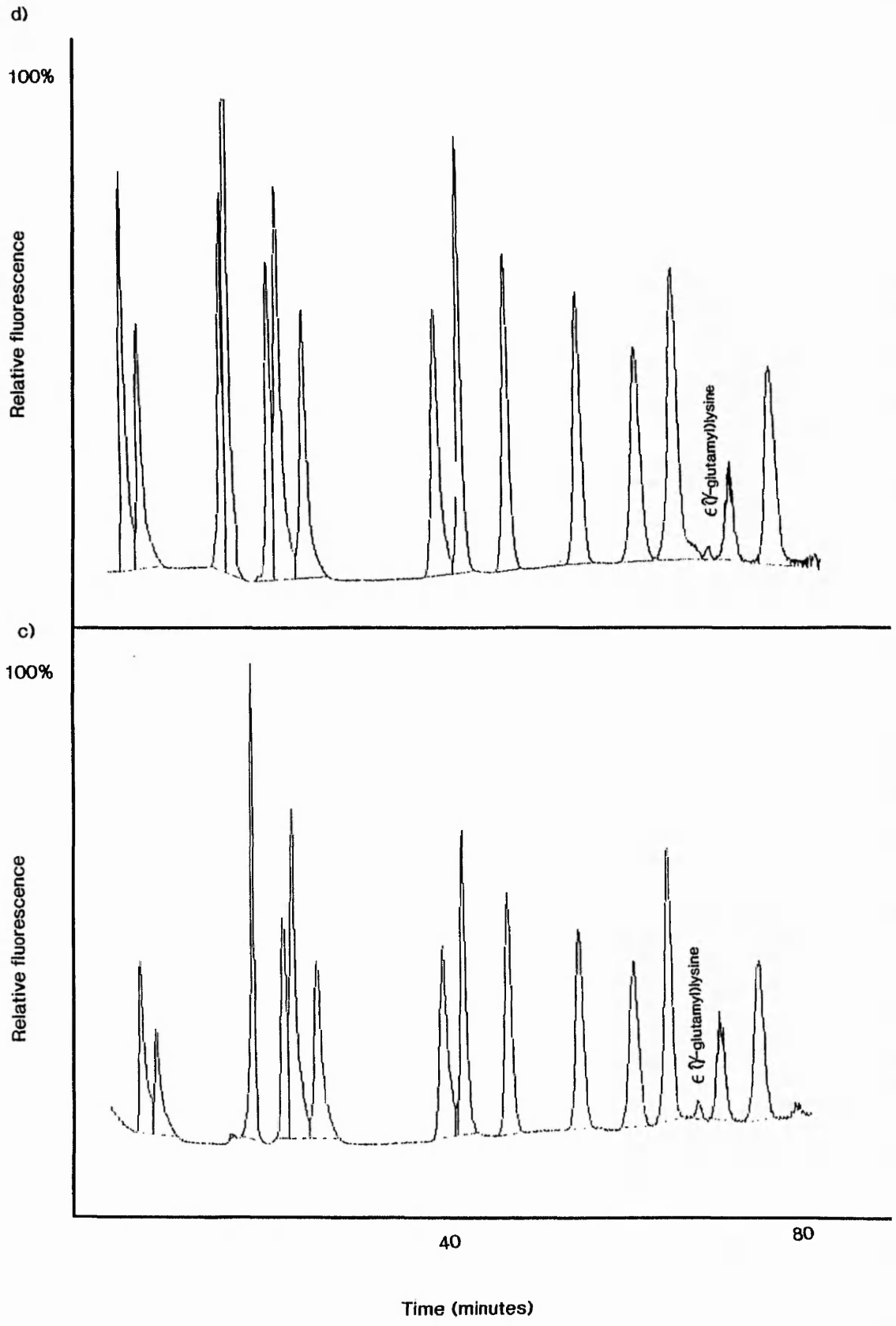
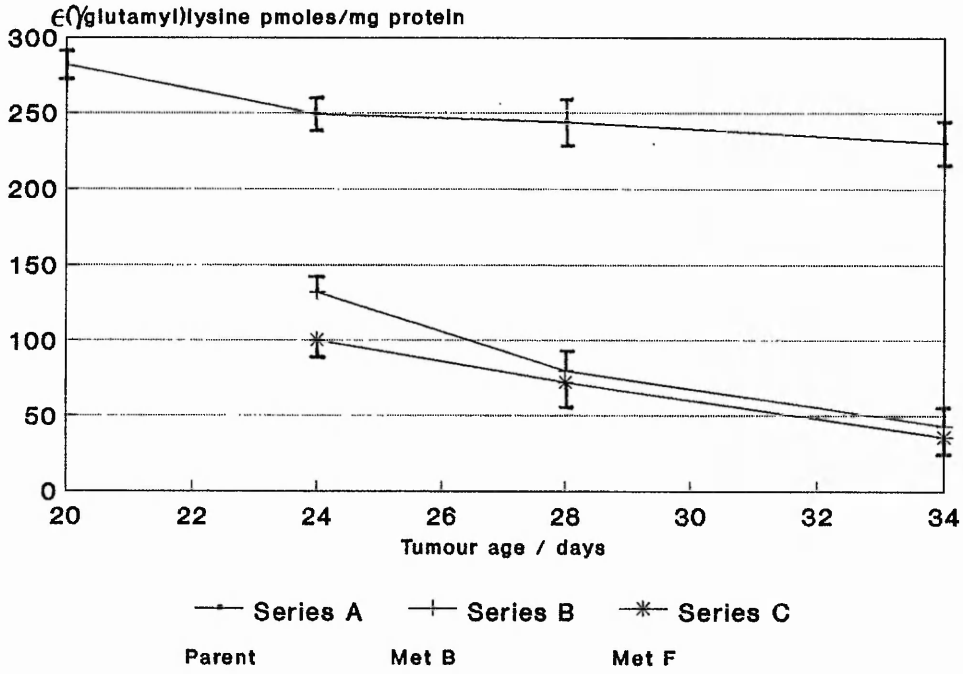

d)
100%
Relative fluorescence
ε(γ-glutamyl)lysine
c)
100%
Relative fluorescence
ε(γ-glutamyl)lysine
40
80
Time (minutes)

Fig. 3.7 : The level of $\epsilon(\gamma$-glutamyl)lysine in hamster fibrosarcomas during growth.

ϵ (γ-glutamyl)lysine levels in digested tumour homogenates were assessed by reverse phase HPLC (sections 2.6.2; 2.8.5; Fig.3.6).

Data represents the mean of 3 experiments ± SE (3 repeats per experiment).


ε(γglutamyl)lysine pmoles/mg protein
300
250
200
150
100
50
0
20
22
24
26
28
30
32
34
Tumour age / days
Series A
Series B
Series C
Parent
Met B
Met F

3.4 IS TRANSGLUTAMINASE INVOLVED IN PROGRAMMED CELL DEATH?

A number of recent reports have suggested that tissue transglutaminase may be involved in the formation of apoptotic bodies, through the crosslinking of cellular proteins, during programmed cell death (Fesus et al, 1987, 1989; Schmidt et al, 1988). Since the metastatic variants and the normal BHK cells provide a model whereby cytosolic transglutaminase activity decreases, with the concomitant decrease in $\epsilon(\gamma$-glutamyl)lysine, it was possible to confirm and extend these reports by assessing the incidence of apoptosis in the various cultured cell lines. In order to compare the apoptotic index (percentage of cells undergoing apoptosis) of the different cell lines, apoptotic bodies were isolated from cells in culture.

3.4.1 Quantitation of apoptotic bodies in cultured cells.

Apoptotic bodies (Fig. 3.8) were isolated from cells using an adaptation of the method Schmidt et al (1988) (section 2.2.5). The normal BHK cell line demonstrated the highest apoptotic index at approximately double that for the weakly metastatic cell lines Met D, Met C and parent, which were themselves greater than for the highly metastatic cell lines Met B and Met E. The highly metastatic cell line, Met F, which demonstrates relatively high levels of cytosolic transglutaminase activity in culture, also demonstrated an apoptotic index more consistent with that of a weakly metastatic cell line (Table 3.7). The coefficient of correlation between cytosolic transglutaminase activity and the apoptotic index is $r = 0.98$.

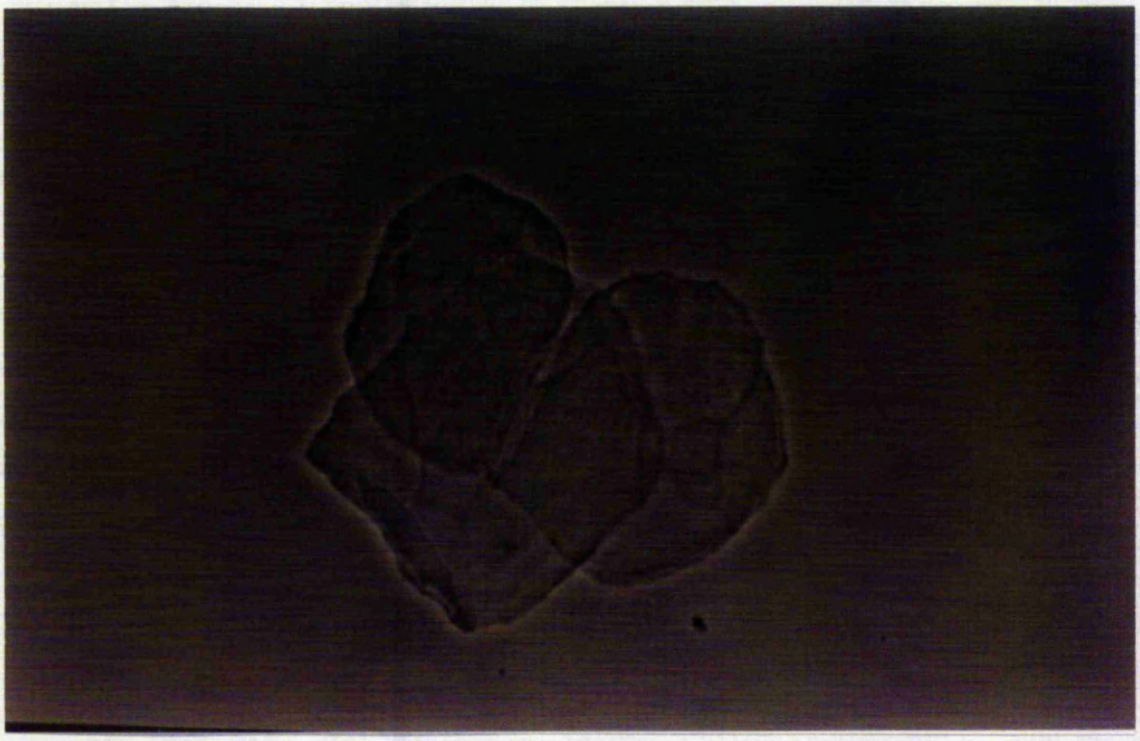
Table 3.7 : Apoptotic indices for cultured cells.

Cell line	Metastatic potential	Apoptotic index
BHK	0	0.048
Met D	1/20	0.025
Met C	4/20	0.021
Parent	6/19	0.018
Met B	20/20	0.009
Met E	19/19	0.011
Met F	20/20	0.020

Apoptotic indices were calculated by counting isolated apoptotic bodies (Fig. 3.8), as described in section 2.2.5, from 2 x 10^7 cells, and are expressed as a percentage of the number of cells from which the apoptotic bodies were isolated.

Fig. 3.8 : Phase contrast micrographs of isolated 'apoptotic bodies'.

Apoptotic bodies were isolated from 10^7 cells by boiling in medium containing 2% (w/v) SDS as in section 2.2.5. The isolated structures were photographed under phase contrast microscopy at a magnification of x5000.



3.4.2 ε (γ-glutamyl)lysine levels in cultured hamster fibrosarcoma cells and isolated apoptotic bodies.

A shortened protocol for proteolytic digestion was used for digesting cells and isolated apoptotic bodies. This was as detailed in section 2.6.2.1 except that cell homogenates and apoptotic bodies were treated directly with 0.15mg proteinase K (in 5mM Tris pH7.4, 0.1% (w/final volume) SDS per mg protein (Maniatis et al, 1982) at 37°C for 12h instead of the three subtilisin steps.

Again, as cytosolic transglutaminase activity decreased the total amount of crosslink present also decreased. Levels of crosslink found in isolated apoptotic bodies were similar to those found in the corresponding number of whole cells (Table 3.8). When ε(γ-glutamyl) lysine levels were expressed as mmoles per 10^6g protein (Table 3.8), it became apparent that apoptotic bodies were indeed highly crosslinked structures, and suggested that the ε(γ-glutamyl)lysine protein crosslink may not be greatly involved in any other cellular process of the fibroblast.

Table 3.8 : Level of $\epsilon(\gamma$-glutamyl)lysine in whole cells, isolated apoptotic bodies and non-apoptotic cells.

Cell line	$\epsilon(\gamma$-glutamyl)lysine			
	nmoles / 10^8cells		mmoles / 10^6g protein	
	whole cells	apoptotic bodies	apoptotic bodies	non-apoptotic cells
Parent	5.09±0.56	4.47±0.49	8940±511	177±24
Met E	1.99±0.32	1.58±0.24	7021±683	89±29
Met F	3.81±0.17	2.89±0.14	8128±467	228±36

Apoptotic bodies were isolated from 10^8 cells as in section 2.2.5. Apoptotic bodies and cells (10^8) were digested sequentially by proteinase K (0.15mg in 5mM Tris, 1% (w/final volume) SDS), pronase, leucine aminopeptidase and carboxypeptidase Y (as detailed in section 2.6.2.1). $\epsilon(\gamma$-glutamyl)lysine was quantitated as in section 2.6.2. The level of $\epsilon(\gamma$-glutamyl)lysine in non-apoptotic cells was estimated by subtracting the apoptotic body value from the whole cell value.

Data represent mean values ± SE of three repeats.

3.5 Transglutaminase activity and apoptosis in B16 mouse melanoma variants.

A brief investigation was carried out into the distribution of transglutaminase, and the level of apoptosis, in the cultured mouse melanocyte lines Melan A (control), B16 F1 (weakly metastatic) and B16 F10 (highly metastatic). Experimental procedures were identical to those used previously for assessing the distribution of enzyme activity and the level of apoptosis in the hamster fibrosarcoma variants.

As with the hamster fibrosarcoma cell lines, the results indicated an inverse relationship between cytosolic transglutaminase activity and metastatic potential (Table 3.9). Again, particulate activity remained constant between cell lines, whilst the cytosolic activity found in the highly metastatic B16 F10 cell line was less than 40% of that found in the control Melan A cell line. The apoptotic index also followed the same trend as that seen in the hamster fibrosarcoma variants; the level of apoptosis was inversely related to metastatic potential and directly related to cytosolic transglutaminase activity (Table 3.9).

Table 3.9 : Transglutaminase distribution and apoptotic index for mouse melanoma variants and melanocytes.

Cell Line		Activity $U/10^6$ cells	Apoptotic index
Melan A (0)	Cytosolic	12.1±1.63	0.038
	Particulate	5.1±0.82	
B16 F1 (low)	Cytosolic	8.8±1.44	0.021
	Particulate	4.8±0.31	
B16 F10 (high)	Cytosolic	4.1±0.23	0.008
	Particulate	3.4±0.16	

Enzyme activity was extracted from cell homogenates and separated as detailed in the legend to Fig.3.2a. Transglutaminase activity in the pooled active fractions was quantitated as in section 2.6.1.1.

Apoptotic indices were calculated by counting isolated apoptotic bodies (Fig. 3.8), as described in section 2.2.5, from 2×10^7 cells, and are expressed as a percentage of the number of cells from which the apoptotic bodies were isolated.

The metastatic potential of each cell line is given in parentheses.

Data represents the mean of 3 experiments ± SE.

3.6 DISCUSSION.

Previous investigators have observed that neoplastic tissues contain reduced levels of transglutaminase activity in comparison to their normal counterparts (Birckbichler et al, 1977; Barnes et al, 1985; Delcros et al, 1987; Hand et al, 1988). It has also been noted that transglutaminase activity decreases from normal tissue, through benign or tumour bearing tissue, to malignant tissue (Delcros et al, 1987; Hand et al, 1988); from non-metastasizing to metastasising rhabdomyosarcoma cells (Delcros et al, 1986), and during the growth of a metastatic osteosarcoma (Barnes et al, 1985; Hand et al, 1987). This reduction in expression of transglutaminase activity may prove to be an important event in neoplasia and metastasis. A number of questions, however, still need addressing in order to confirm this important observation.

Measurement of transglutaminase activity in cultured metastatic variants of an HSV-2 induced hamster fibrosarcoma and mouse melanoma variants, indicated that decreases in transglutaminase activity were associated with increasing metastatic potential, suggesting that an inverse relationship might exist between the two parameters. These findings are supported by the recent work of Chung et al, 1990, who have also noted an inverse relationship between transglutaminase activity and metastatic potential using the B16 melanoma model; and Romijn, 1990, who has noted the existence of a similar relationship in prostate cancer cells.

However, one cell line, the highly metastatic Met F, did not conform to this relationship in the artificial environment induced by cell culture. It is realistic to assume that pressures exerted on the

growing tumour by the host animal may influence cellular behaviour, and thus the expression and selection of the metastatic phenotype. This therefore necessitated the measurement of transglutaminase activity in an in vivo situation, in this case, solid tumours derived from the subcutaneous implantation of cells grown in vitro. Although still an artificial system, tumour cells are open to the influence of hormones, growth factors, LAK cells, NK cells and other homeostatic factors not present in culture.

By day 20 of tumour growth, when cells were established in the host environment, the level of transglutaminase activity in the highly metastatic tumour lines, Met B, E and F, was found to be comparable, and significantly lower (approximatly half) than that found in the weakly metastatic tumour lines, parent and Met D. A further interesting observation was that, during the growth and progression of the highly metastatic tumours, enzyme activity continued to decline, such that after 34 days growth, the total transglutaminase activity present in these tumours was only 40% of that found initially. In the weakly metastatic tumour lines, levels of transglutaminase activity did not differ significantly during growth although a slight decline occurred in the parent line. These results, obtained from growing solid tumours, would therefore support the inverse relationship between transglutaminase activity and metastatic potential observed in cultured cell lines, and further, they suggest that reduced transglutaminase activity maybe an important feature of tumour progression.

Crucial to the establishment of the theory that reduced transglutaminase activity is important to the metastasizing phenotype, is the ability to correlate the observed reduction in enzyme activity, measured by an in vitro assay, with reduced amounts of the endogenous

enzyme product $\epsilon(\gamma$-glutamyl)lysine found in the tumours. In the metastatic variants Met B and F, and in the parent tumour, a direct relationship exists between transglutaminase activity as assessed by an in vitro assay, and the level of $\epsilon(\gamma$-glutamyl)lysine present.

In earlier studies undertaken on chemically induced hepatocellular carcinomas (Hand et al, 1988) it was demonstrated that the reduction in expression of transglutaminase activity within these tumours could be attributed to a selective reduction in the activity of the cytosolic form of the enzyme. It was therefore important to establish whether the variation in transglutaminase activity seen in the HSV-2 induced fibrosarcomas, and the decline in activity seen in the two highly metastatic variants during growth, could also be related to a selective reduction in one or other of the two enzyme forms. When comparing the weakly metastatic tumour lines to the highly metastatic tumours, it became clear that the observed differences in enzyme activity were due, in the main, to a selective reduction in the activity of the cytosolic form of transglutaminase. Since these studies were undertaken on cytosolic and particulate enzymes that had been semi-purified by anion-exchange chromatography, any endogenous modulators capable of inhibiting measured enzyme activity, such as polyamines, would most likely have been removed. Thus they also confirm that the differences in total enzyme activity seen between the highly metastatic variants, Met B, E and F, and the weakly metastatic parent and Met D variants, represent true differences in the levels of active enzyme present within these tumours. Although the interrelationship between the two enzyme forms is presently unknown, the close correlation between the levels of activity of the cytosolic enzyme and the amount of the protein crosslink, $\epsilon(\gamma$-glutamyl)lysine,

found in the different tumours suggests that the cytosolic transglutaminase is the major enzyme responsible for this endogenous product, with the particulate enzyme potentially maintaining a basel level of protein crosslinking.

Fesus et al (1989) have suggested that transglutaminase activity may be involved in the process of programmed cell death, and recent work in this laboratory using tissue slices (Hand et al, 1990) has indicated that transglutaminase activity causes the incorporation of radiolabelled amine substrates into a highly crosslinked heavy molecular weight 'polymer' thought to be apoptotic bodies. This work has now been confirmed and extended. Firstly, there is the finding that a direct relationship exists between the cytosolic transglutaminase activity within a cell line and that cell lines apoptotic index. Any cell within the tumour population whose ability to undergo programmed cell death is impaired, will consequently be capable of giving rise to a greater number of progeny by division. Thus as the tumour progresses, a greater proportion of its constituent cells will be unable to enter apoptosis. Since it is likely that these cells will be deficient in cytosolic transglutaminse, this would explain the decrease in cytosolic activity that occurs with tumour growth. Similarly, if, as has been suggested (Fesus, 1990; Piacentini, 1990) cytosolic transglutaminase is only active in those cells undergoing cell death, then the action of NK and LAK cells, thought to kill their targets by inducing apoptosis, could explain the increased levels of cytosolic transglutaminase found in the tumours compared to cultured cells. Secondly, 75 - 85 % of the $\epsilon(\gamma$-glutamyl)lysine present in cells is found within apoptotic bodies indicating these structures to contain highly crosslinked proteins, in comparison to the

proteins in cells not undergoing programmed cell death.

A cells inability to enter programmed cell death will not only prolong its life within a tumour mass, but may also protect it in the circulation from the action of NK and LAK cells. Thus lack of cytosolic transglutaminase activity could enhance the metastatic potential of an individual cell (the ability to disseminate, invade and recolonise) by prolonging its life both by prevention of programmed cell death and protection from killer cell action.

The following chapter undertakes to investigate the molecular events leading to the reduction in cytosolic transglutaminase activity, by assaying the levels of transglutaminase antigen present in both cultured cells and solid tumours.

4: LEVEL AND DISTRIBUTION OF ANTIGENIC TRANSGLUTAMINASE IN METASTATIC VARIANTS OF AN HSV-2 INDUCED HAMSTER FIBROSARCOMA.

4.1 INTRODUCTION.

In the previous chapter, it was noted that an inverse relationship existed between metastatic potential and cytosolic transglutaminase activity, and that further reductions in the activity of the cytosolic form occurred during the progression of highly metastatic tumours. This chapter addresses the question of what causes this reduction in cytosolic activity; is it a reduction in the expression of the enzyme protein, or a reduction of the activity of the enzyme itself. This has been achieved by ascertaining the level of expression of all antigenic forms of transglutaminase present, both in cultured cells and in the solid tumours derived from those cells.

4.2 DEVELOPMENT OF A QUANTITATIVE ASSAY FOR TRANSGLUTAMINASE PROTEIN.

The determination of the concentration of a single protein in a complex mixture of proteins can be achieved by utilising the specific binding of antigen to antibody in an immunoassay. This would obviate the purification of the protein prior to estimating its concentration. Various immunoassay techniques are available, such as radioimmune assays and fluorescence immunoassays, but for simplicity and economy, it was decided to develop an enzyme linked immunosorbant assay (ELISA) for transglutaminase. Two polyclonal anti-cytosolic transglutaminase antibodies were available, one (courtesy of Dr.P.J.Davies, Dept. of Pharmocology, University of Texas Medical School, Houston, TX., U.S.A.) raised against guinea pig liver cytosolic transglutaminase in goat, and affinity purified against guinea pig liver cytosolic transglutaminase (Goat anti-gplt), and the other (courtesy of Dr.D.Hand, Dept. of Life Sciences, Nottingham Polytechnic, Nottingham, U.K.) raised in rabbit, against rat liver cytosolic transglutaminase (Rabbit anti-rlct). As a standard, and also for determining optimum assay conditions, guinea pig liver cytosolic transglutaminase, purified by Dr.D.Hand by the method of Connelan et al (1971), was used.

4.2.1 Development of a sandwich ELISA.

Preliminary non-competitive ELISA's, where 1 μg of transglutaminase antigen per well was bound directly to the plastic microtitre plate, produced poor titration curves not suitable for

assay, when using either the goat or the rabbit polyclonal antibody (Fig.4.1), suggesting that the binding of transglutaminase to the plastic plate might be poor. Since antibodies to cytosolic transglutaminase were available from two different species, the potential existed for the development of an assay in which transglutaminase antigen was captured by an antibody already bound to the plate and then detected with a second antibody, thus 'sandwiching' the antigen. Using this system, with either the goat antibody as the capture and the rabbit antibody as the indicator or vice versa, well defined titration curves were obtained (Fig.4.2).

4.2.2 Optimisation of assay conditions.

To assess the optimum dilution for each antibody in the assay, and to ascertain the most favourable order in which to use the antibodies, two sets of plates were set up :-

Set A :

capture - affinity purified goat anti-gplt antibody.

indicator - rabbit anti-rlct antibody.

Set B :

capture - rabbit anti-rlct antibody.

indicator - affinity purified goat anti-gplt antibody.

Plate 1 :

capture antibody diluted 1/500

antigen at 1 ug per well

indicator antibody diluted :- 1/100, 1/500, 1/1000, 1/2500, 1/5000,

1/7500, 1/10 000, 1/25 000.

Plate 2 :

capture antibody diluted :- 1/100, 1/500, 1/1000, 1/2500, 1/5000, 1/7500, 1/10 000, 1/25 000.

antigen at 1 μg per well

indicator antibody diluted 1/500.

Lanes were set up in triplicate, and controls included for capture antibody, antigen and indicator antibody.

From these plates it was apparent that no advantage would be gained by having one antibody as the capture antibody as opposed to the other. Since the first antibody would be required to trap antigen from a solution of mixed proteins, and would therefore need to be more specific, it was decided to use the affinity purified goat anti-gplt antibody as the capture antibody. Dilutions of 1/1000 and 1/4000, for rabbit anti-rlct and goat anti-gplt respectivly, produced 50% saturation of transglutaminase at 1 ug per well (Fig.4.2), and these dilutions were adopted for use in the assay.

To test the sensitivity of the assay, triplicate dilutions of purified guinea pig liver transglutaminase and immunoaffinity purified hamster fibrosarcoma cytosolic transglutaminase (refer to section 6.2.3), ranging from 1 μg to 250 pg per well, were bound by a 1/4000 dilution of goat anti-gplt, with a 1/1000 dilution of rabbit anti-rlt used as the indicator antibody. The assay produced equivalent linearity between 2.5 ng and 350 ng (Fig.4.3). Thus a quantitative sandwich ELISA was developed that was suitable for assaying levels of transglutaminase between 625 pM and 87.5 nM.

Figure 4.1 : Non-competitive ELISA for the determination of IgG reactivity.

Determination of a 50% saturating concentration of Goat anti-guinea pig liver cytosolic transglutaminase IgG by titration against an optimal amount (lug) of guinea pig liver cytosolic transglutaminase.

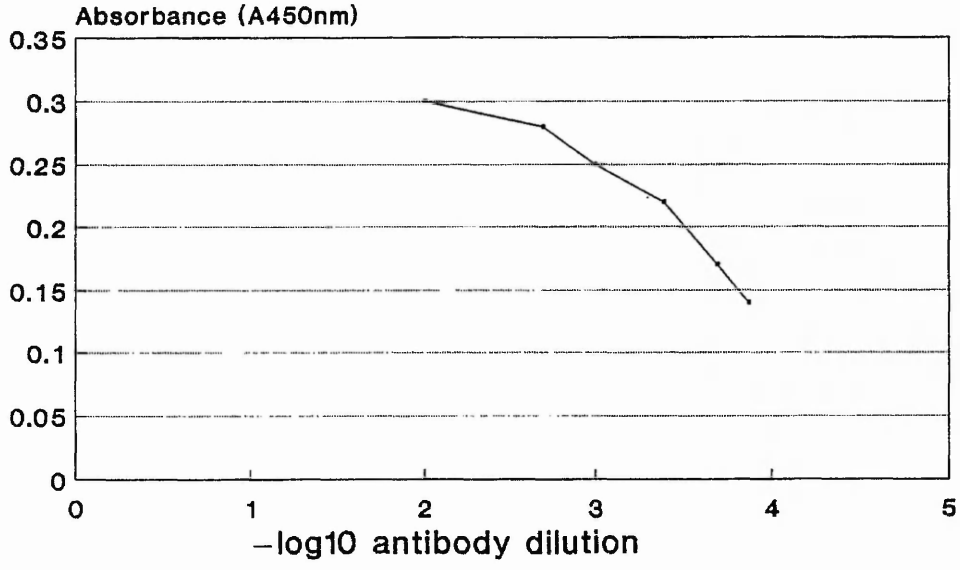

Absorbance (A450nm)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
1
2
3
4
5
−log10 antibody dilution

Figure 4.2 : Optimisation of antibody dilutions for the sandwich ELISA.

In order to optimise the sandwich ELISA protocol (section 2.6.3.1), assay plates were set up as follows :-

A1 : capture antibody - 1/500 dilution of goat anti-gplt antibody.
indicator antibody - rabbit anti-rlct titrated against lug guinea pig liver cytosolic transglutaminase.

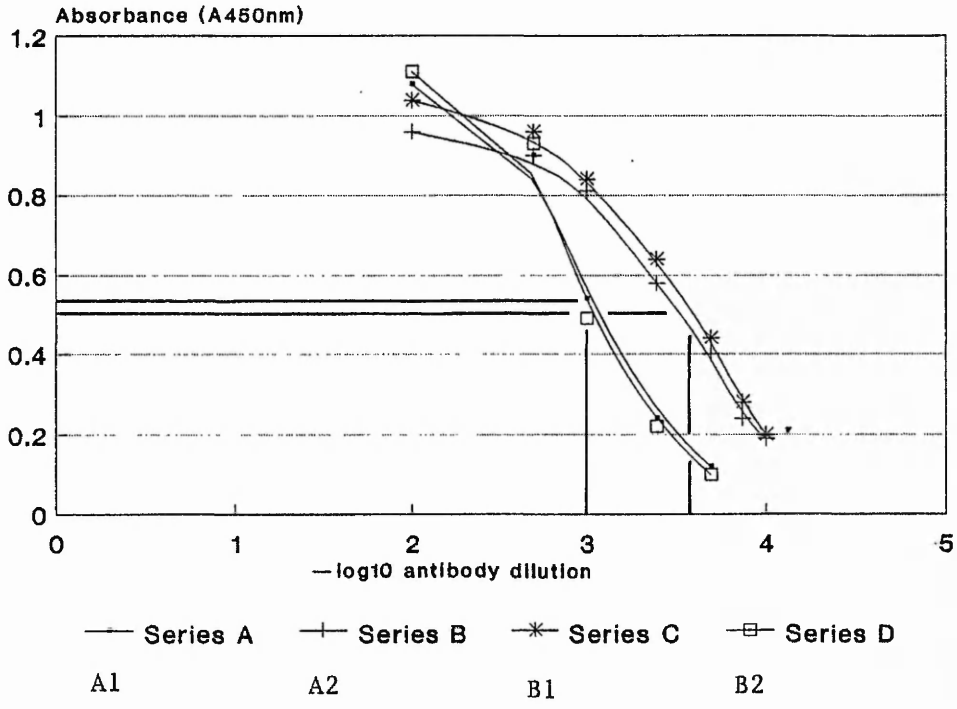
A2 : capture antibody - goat anti-gplt antibody titrated against lug of guinea pig liver cytosolic transglutaminase.
indicator antibody - 1/500 rabbit anti-rlct antibody.

B1 : capture antibody - 1/500 dilution of rabbit anti-rlct antibody.
indicator antibody - goat anti-gplt titrated against lug of guinea pig liver cytosolic transglutaminase.

B2 : capture antibody - rabbit anti-rlct antibody titrated against lug of guinea pig liver cytosolic transglutaminase.
indicator antibody - 1/500 dilution of goat anti-gplt antibody.

Solid lines indicate 50% saturation of antigen.

Optimisation of assay conditions

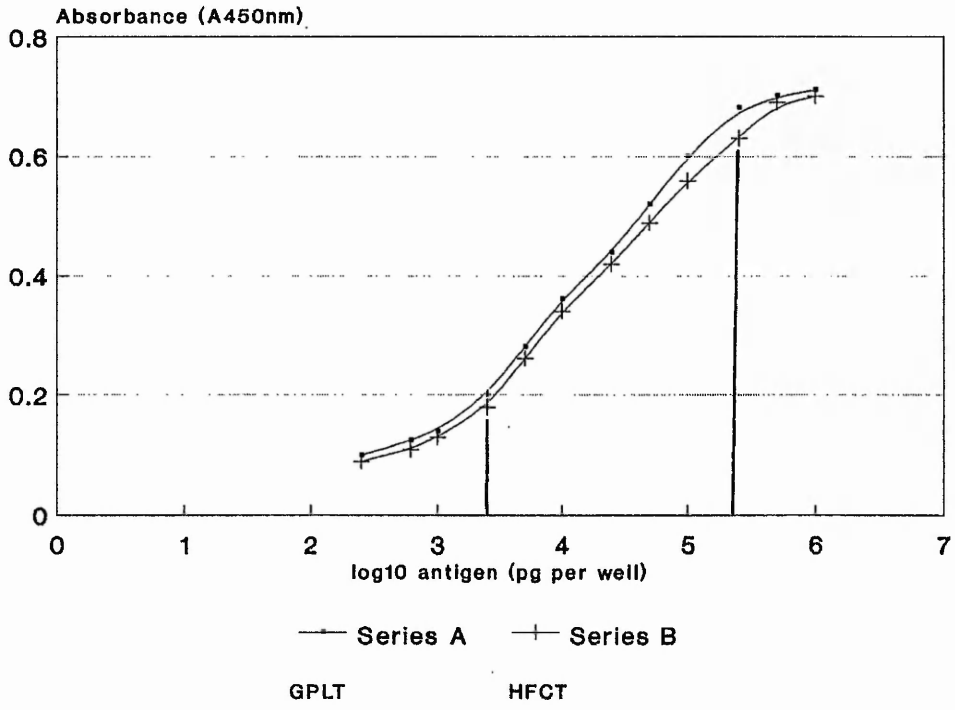


A1 A2 B1 B2

Figure 4.3 : Optimisation of standard curve for quantitative sandwich ELISA.

To test the sensitivity of the assay, and to produce a suitable standard curve to use in assays, triplicate dilutions (1ug - 250pg) of guinea pig liver cytosolic transglutaminase and hamster fibrosarcoma cytosolic transglutaminase were introduced into the optimised assay (section 2.6.3.1).

Solid lines indicate the region of equivalent linearity.


Absorbance (A450nm)
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
7
log10 antigen (pg per well)
Series A
Series B
GPLT
HFCT

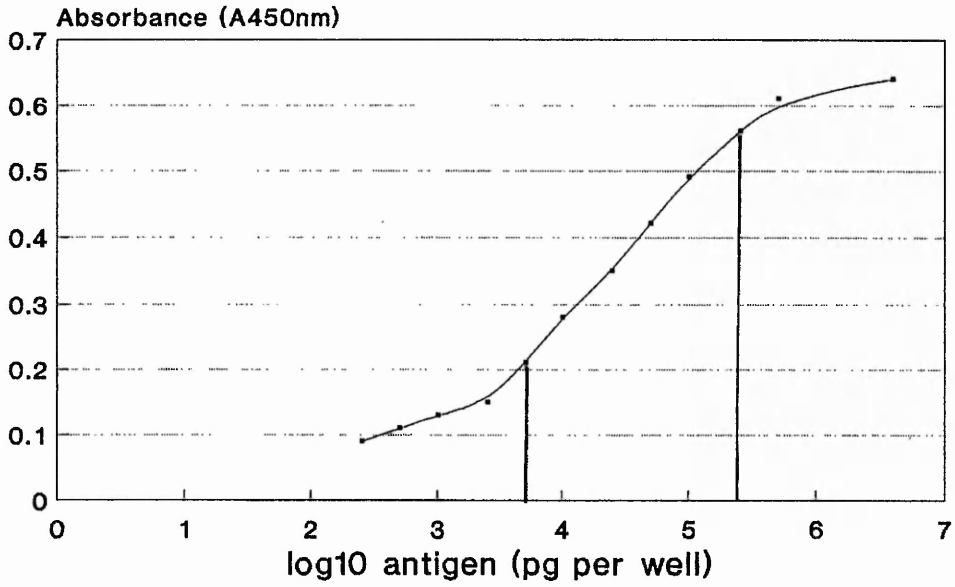
4.2.3 Poly-1-lysine ELISA for transglutaminase.

Only a limited amount of affinity purified goat anti-gplt antibody was available, and so an assay was devised in which antigen was bound to the microtitre plate by poly-1-lysine (section 2.6.3.2). A 1/1000 dilution of rabbit anti-rlct was then used as before to indicate the presence of transglutaminase. This assay was used as a method of detecting transglutaminase in the eluent fractions collected after liquid chromatography, but not for quantitating the concentration of transglutaminase. Linearity was achieved between 5 and 200 ng (Fig.4.4), and therefore the assay is neither as sensitive nor as accurate as the sandwich ELISA.

Figure 4.4 : Standard curve for poly-l-lysine ELISA.

Dilutions (lug - 250pg) of cytoslic transglutaminase from guinea pig liver or hamster fibrosarcoma were bound to the assay plate by poly-l-lysine (50ug per well) and visualised by a 1/1000 dilution of rabbit anti-rlct antibody (section 2.6.3.2).

Solid lines indicate the region of linearity.


Absorbance (A450nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
7
log10 antigen (pg per well)

4.3 LEVELS OF CYTOSOLIC TRANSGLUTAMINASE ANTIGEN IN METASTATIC VARIANTS OF AN HSV-2 INDUCED HAMSTER FIBROSARCOMA.

Changes occurring in the levels of activity of an enzyme in a tissue are not necessarily a true reflection of the expression of that enzyme, the regulation of which may undergo a number of events both post-transcriptional and post-translational. Hence it was necessary to evaluate the expression of cytosolic transglutaminase protein. To this end, cytosolic particle-free supernatants (71,000 gav), from tumour tissue homogenized in buffer A (section 2.4.3), were diluted appropriatly in PBS and then assayed for transglutaminase antigen by a quantitative sandwich ELISA (section 2.6.3.1).

4.3.1 During tumour growth.

Tumours from the parent, Met B, D, E and F cell lines were assayed for cytosolic transglutaminase antigen at different time points during their growth. It was found that there was no significant difference between the level of transglutaminase antigen expressed in each tumour, regardless of age or metastatic potential (Table 4.1). Since there is a reduced amount of the cytosolic transglutaminase activity found in the highly metastatic variants when compared with the weakly metastatic Met D and parent tumours, with a further drop in the activity of this enzyme occurring during the growth of the highly metastatic tumours, this leads to the conclusion that alterations in the specific activity of the cytosolic enzyme (units/ng transglutaminase antigen) are occurring. Thus the initial specific

activity of the cytosolic transglutaminase present in the highly metastatic lines was significantly less than that found in the weakly metastatic lines, and during tumour growth (24 - 34 days) this decreased by a further 30 - 40%. In contrast, specific activities in the tumours derived from the Met D and parent lines did not alter significantly during tumour growth (Table 4.2).

Table 4.1 : Levels of transglutaminase antigen in the cytosol of HSV-2 induced hamster fibrosarcomas during tumour growth.

Variant	Transglutaminase ng/ug DNA			
	Day 20	Day 24	Day 28	Day 34
Met D (1/20)	6.5±0.30			6.7±0.28
Parent (6/19)	6.3±0.24	7.0±0.25	5.7±0.31	6.6±0.28
Met B (20/20)		6.8±0.33	6.2±0.19	6.4±0.34
Met E (19/19)	6.8±0.32			6.5±0.33
Met F (20/20)		6.9±0.41	6.4±0.25	6.3±0.27

Tumours were harvested at the days shown. Non-necrotic tissue (200 - 500 mg) was homogenised (section 2.4.1) to give a 20% (w/v) homogenate which was then separated into a cytosolic fraction and a 71,000g pellet by centrifugation (section 2.4.3). Antigen concentration was quantitated by a sandwich ELISA (section 2.6.3.1).

The figures in parentheses show metastatic potential.

Data represents the mean values of three separate experiments ± SE.

Table 4.2 : Specific activity of transglutaminase in the cytosol of HSV-2 induced hamster fibrosarcomas during tumour growth.

Variant	Specific activity U/ng transglutaminase.			
	Day 20	Day 24	Day 28	Day 34
Met D (1/20)	0.22±0.034			0.21±0.20
Parent (6/19)	0.18±0.021	0.15±0.029	0.18±0.012	0.15±0.020
Met B (20/20)		0.09±0.011	0.05±0.008	0.03±0.003
Met E (19/19)	0.10±0.038			0.03±0.008
Met F (20/20)		0.06±0.005	0.04±0.008	0.02±0.004

Tumours were harvested at the days shown. Non-necrotic tissue (200 - 500 mg) was homogenised (section 2.4.1) to give a 20% (w/v) homogenate which was then separated into a cytosolic fraction and a 71,000g pellet by centrifugation (section 2.4.3). Antigen concentration was quantitated by a sandwich ELISA (section 2.6.3.1). Activity was measured by the Ca^{2+} dependent incorporation of ^{14}C-putrescine into N´N-dimethylcasein (section 2.6.1.1).

The figures in parentheses show metastatic potential.

Data represents the mean values of three separate experiments ± SE.

4.4 DISTRIBUTION OF ANTIGENIC TRANSGLUTAMINASE IN CULTURED METASTATIC VARIANTS OF AN HSV-2 INDUCED HAMSTER FIBROSARCOMA AND GROWING TUMOURS DERIVED FROM THOSE CELLS.

In order to confirm that the level of antigen found in the cytosolic fraction of tumour homogenates was due solely to cytosolic transglutaminase, and not a combination of the cytoslic form and other contaminating forms of transglutaminase, the specificity of the antibodies was assessed. Eluent fractions from the anion-exchange chromatography (Mono-Q column) of non-ionic detergent extracts of tumour tissue, and the plasma transglutaminase Factor XIII (Boehringer), were assessed by the quantitative sandwich ELISA for cross-reactivity with the anti-cytosolic transglutaminase antibodies. From this it was apparent that, whilst Factor XIII did not cross-react with either one or both of the anti-cytosolic transglutaminase antibodies, the assay was not specific for the cytosolic form of tissue transglutaminse. Three antigenic peaks were apparent in the elution profiles obtained, one corresponding to particulate activity, one corresponding to cytosolic activity and one eluting between the two. This evidence necessitated firstly, the evaluation of the affinity of the antibodies, under the conditions of the sandwich ELISA, for particulate transglutaminase and the inactive antigenic 'transglutaminase', and secondly, the assessment of antigen distribution in growing tumours and cultured cells.

Antigen profiles, of the eluent from the anion-exchange chromatography of detergent extracts, were obtained using the poly-l-lysine ELISA, and the pooled peaks assayed for antigen concentration using the sandwich ELISA.

Purification of the particulate and inactive forms of tissue transglutaminase was undertaken (refer to section 6.2.1) and the affinity of the quantitative ELISA assessed for each.

4.4.1 Presence of a third antigenic, but inactive, form of transglutaminase.

All forms of tissue transglutaminase were extracted, with non-ionic detergent, from cell and tumour homogenates (section 2.4.2), and separated by anion-exchange chromatography using a gradient of 0 - 0.5 M NaCl in Tris/EDTA as the elution buffer (section 2.8.1.2). Eluent fractions were assayed initially using a poly-l-lysine ELISA to give antigen profiles. Three distinct peaks of antigenicity were demonstrated in the metastatic sublines; one betwen 0.15 - 0.25 M NaCl (corresponding to particulate activity); one between 0.35 - 0.43 M NaCl (corresponding to cytosolic activity); and the third between 0.28 - 0.33 M NaCl (Fig.4.5). However, only two antigenic peaks, corresponding to the particulate and cytosolic activity peaks, were present in the 'normal' hamster fibroblast cell line (Fig.4.5).

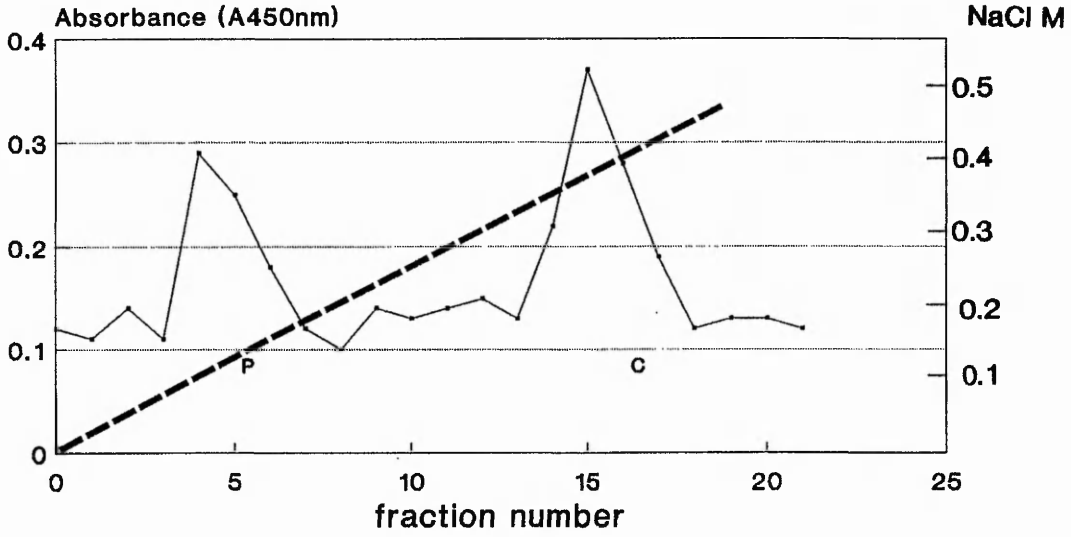
4.4.2 Sandwich ELISA affinity for particulate and inactive antigenic transglutaminase.

Microtitre assay plates were set up as for Set A in section 4.2.2, but including purified particulate transglutaminase, purified inactive antigen (refer to section 6.2.1) and Factor XIII instead of cytosolic transglutaminase. Dilutions of 1/1000 and 1/2050 for the

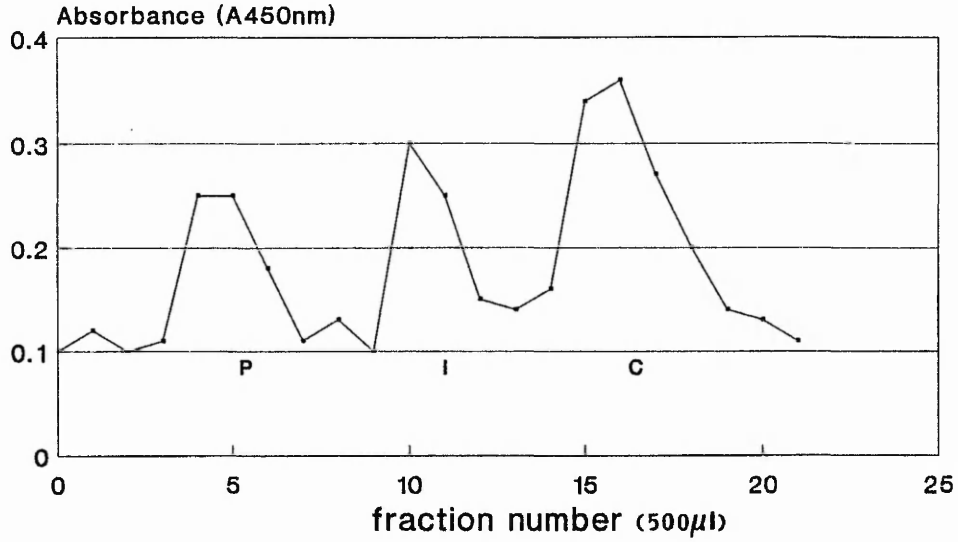
Figure 4.5 : Typical anion-exchange chromatography elution profiles of transglutaminase antigen from cultured fibrosarcoma variants.

Cells were harvested by incubating for 5min in 5mM Tris, 2mM EDTA (TE), pelleted gently in an MSE Centaur bench top centrifuge (1500 rpm), washed twice with TE, resuspended in 600ul (10^7 cells per 300ul) of cold homogenising buffer and then homogenised (section 2.4.1). The homogenates were then separated into a 71,000g pellet and a cytosol fraction by centrifugation. The pellet was then extracted with non-ionic detergent by resuspension of the pellet in buffer containing 1% (w/v) Lubrol-PX and being left on ice for 1h before being centrifuged to give a particle free supernatant (PFS) and a 71,000g pellet (section 2.4.2). Proteins (~5mg) in the PFS were separated by anion-exchange chromatography on a Pharmacia Mono-Q column (1ml) using a 0 - 0.5 M NaCl gradient (section 2.8.1.1). The fractions obtained were assayed for transglutaminase antigen by a poly-l-lysine ELISA (section 2.6.3.2) to give an antigen profile.

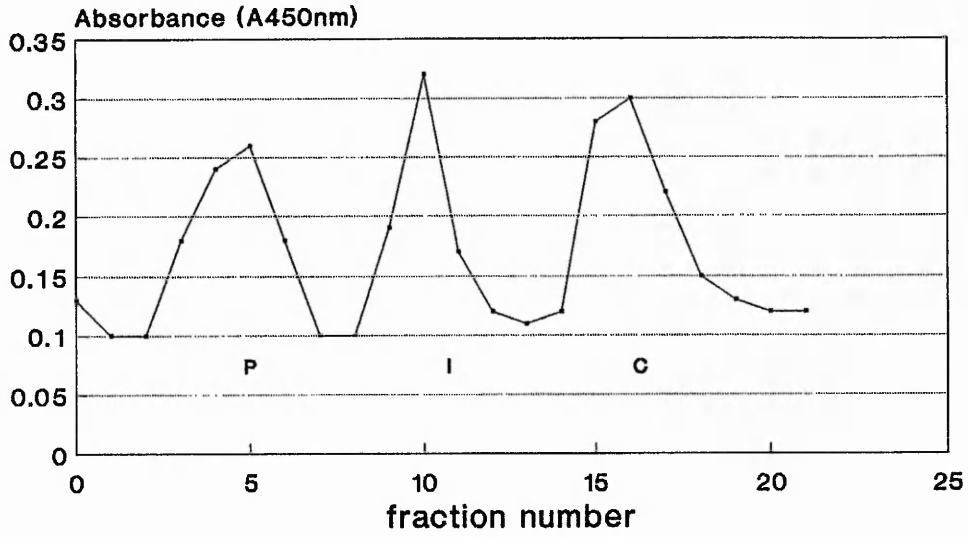
NaCl gradient is indicated by a dotted line.



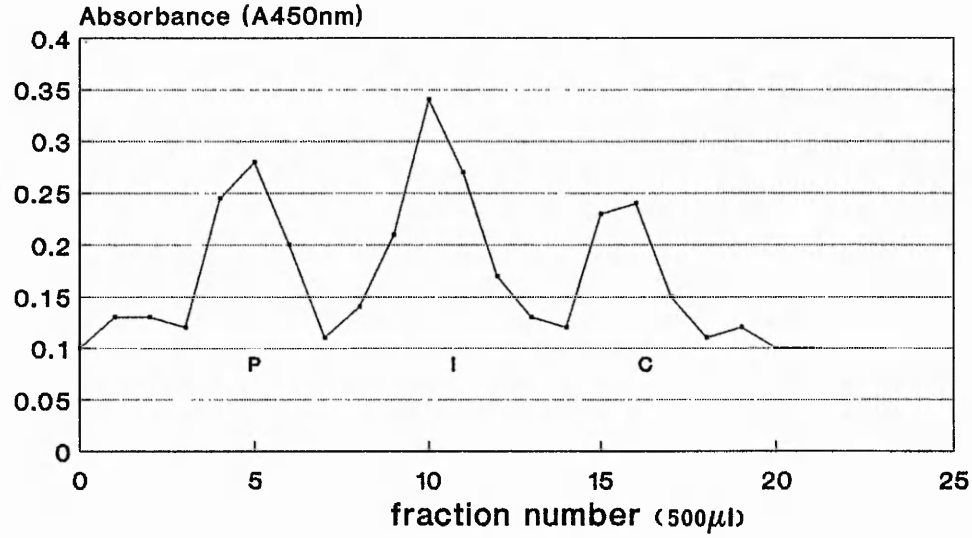
Antigen profile from anion exchange chromatography, BHK.



Antigen profile from anion exchange chromatography, Met D.



Antigen profile from anion exchange chromatography, Parent.



Antigen profile from anion exchange chromatography, Met B.

rabbit and goat antibodies respectively, gave 50% saturation of particulate antigen at 1 ug per well (Fig.4.6). This suggests the functional affinity of the rabbit anti-rlct for the two active forms of tissue transglutaminase is similar, whilst that of the goat anti-gplt is higher for cytosolic transglutaminase than for the particulate form. Dilutions of 1/2000 and 1/5000 gave 50% saturation of the inactive antigen for the rabbit anti-rlct and the goat anti-gplt respectively (Fig.4.6). This suggests that the functional affinity of both the rabbit anti-rlct and the goat anti-gplt for the inactive form of tissue transglutaminase is higher than that for the cytosolic and particulate forms.

4.4.3 Comparison of the sensitivity of the sandwich ELISA for the three antigenic forms of transglutaminase.

To compare the sensitivity of the assay for the particulate, cytosolic and inactive transglutaminases, triplicate dilutions of each antigen form, purified from hamster fibrosarcomas (refer to section 6.2.1), and purified guinea pig liver transglutaminase, ranging from lug to 250 pg per well, were introduced into the standard sandwich assay. Equivalent linearity was produced for the hamster particulate and cytosolic transglutaminases and the guinea-pig standard, between 2.5 and 350 ng per well (Fig.4.7); linearity for the inactive antigen occurred betwen 2 and 175 ng per well, but the plot was displaced to the left of that for the standard and the two active forms (Fig.4.7), confirming that the affinity of the two antibodies for the inactive antigen is greater than that for the particulate and cytosolic forms.

In order to be able to quantitate inactive transglutaminase

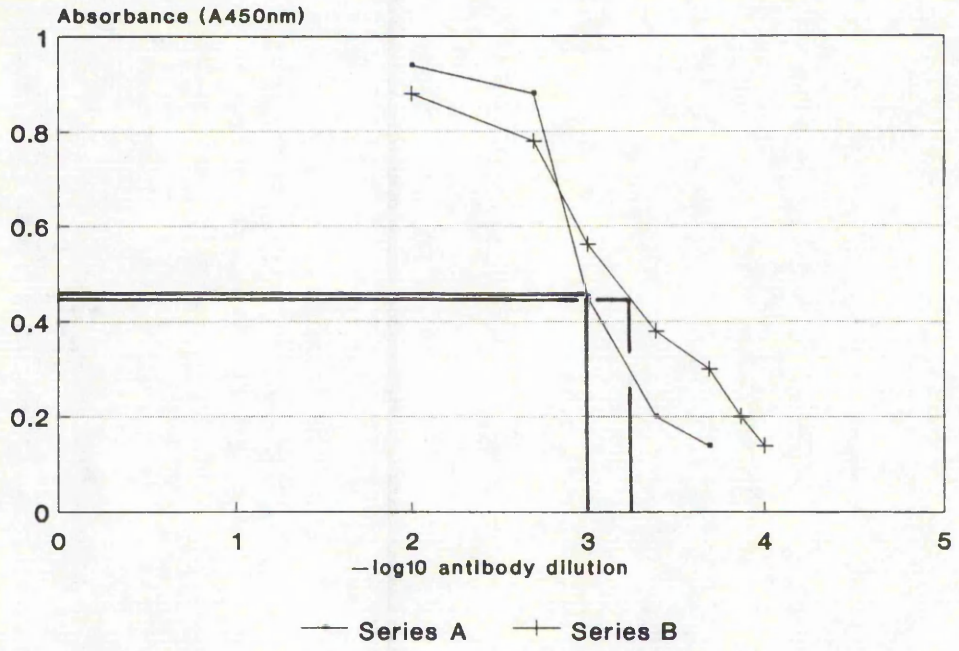
Figure 4.6 : Optimisation of antibody dilutions for the sandwich ELISA with reference to particulate, inactive tissue transglutaminase and Factor XIII.

In order to optimise the sandwich ELISA protocol (section 2.6.3.1) for particulate and inactive transglutaminase, assay plates were set up as for Figure 4.2, using particulate and inactive transglutaminase purified from the P8 rat sarcoma (section 6.2.1) and commercial Factor XIII (Boehringer).

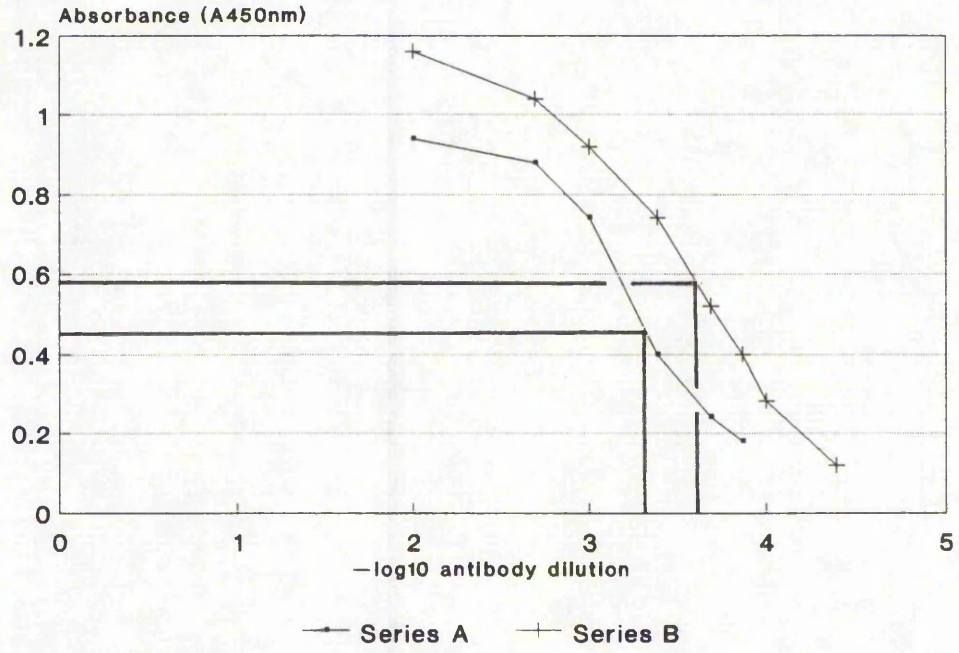
Series A :- Rabbit anti-rat liver cytosolic transglutaminase IgG titrated against lug of antigen bound to the assay plate by a 1/1000 dilution of goat anti-guinea pig liver cytosolic transglutaminase, as in section 2.6.3.1

Series B :- Goat anti-guinea pig liver cytosolic transglutaminase IgG titrated against lug of antigen bound to the assay plate by a 1/1000 dilution of rabbit anti-rat liver cytosolic transglutaminase, as in section 2.6.3.1

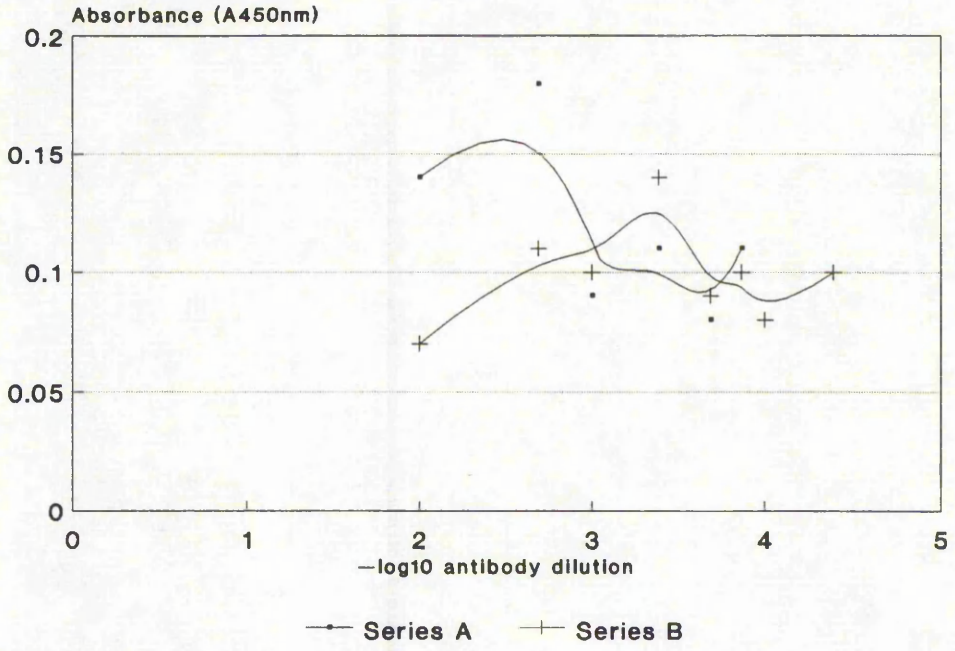
Solid lines indicate 50% saturation of antigen.



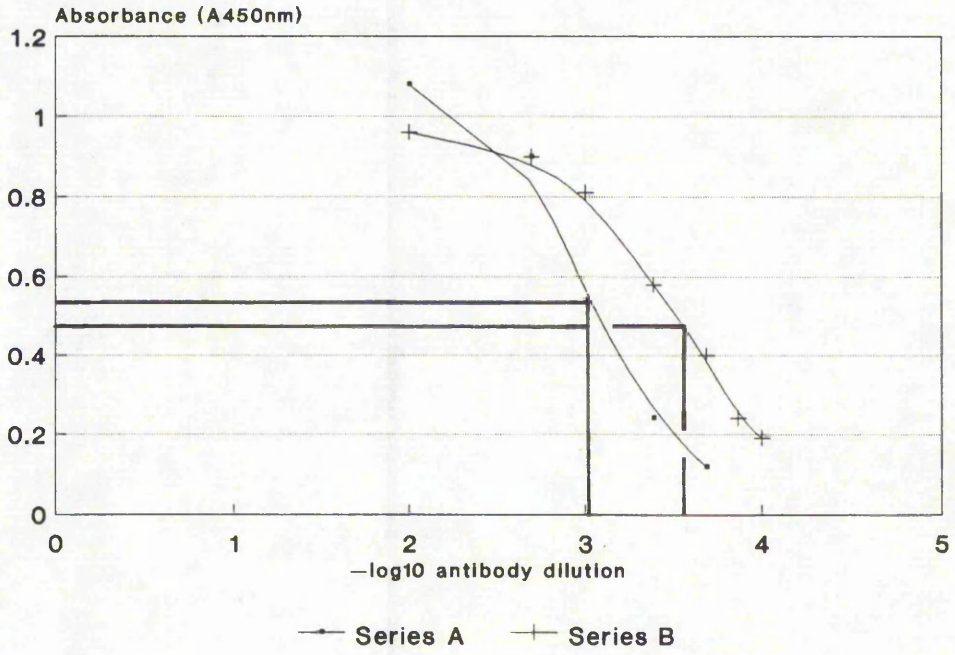
Particulate; A=goat, B=rabbit.



Inactive; A=goat, B=rabbit.



Factor XIII; A=goat, B=rabbit.



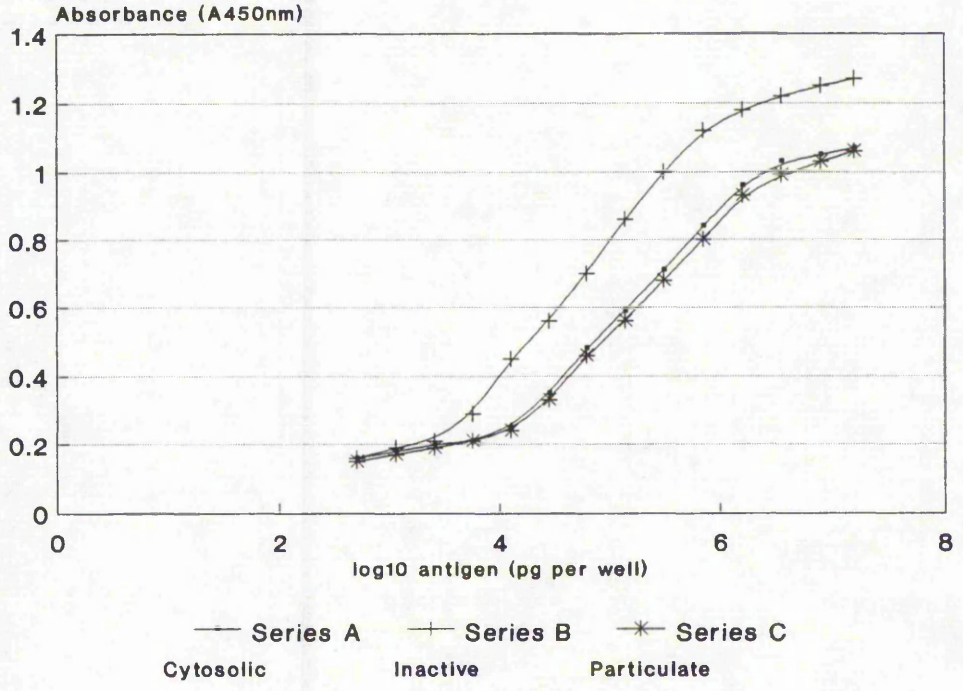
Cytosolic; A=goat, B=rabbit.

Figure 4.7 : Standard curves for the three forms of transglutaminase in the quantitative sandwich ELISA.

To test and compare the sensitivity of the assay for the three different transglutaminase forms, triplicate dilutions (1ug - 250pg) of each form were introduced into the optimised assay (section 2.6.3.1).

Cytosolic transglutaminase was purified from guinea pig liver (courtesy of Dr. D. Hand, Nottingham Polytechnic).

Particulate and inactive transglutaminase forms were purified from rat sarcomas (section 6.2.1).


Absorbance (A450nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
log10 antigen (pg per well)
Series A
Cytosolic
Series B
Inactive
Series C
Particulate

from a standard curve derived from guinea-pig liver cytosolic transglutaminase (purified inactive transglutaminase was not available in large enough quantities to enable its regular use as a standard for quantitative assays) a conversion formula was calculated :-

$$(\text{inactive } A_{450nm} \times 0.92) - 0.05 = \text{cytosolic } A_{450nm}$$

4.4.4 Measurement of the three antigenic forms of transglutaminase.

The total concentration of transglutaminase antigen, in pooled fractions (from anion-exchange chromatography), of the particulate, inactive and cytosolic forms was assessed by quantitative sandwich ELISA (Table 4.3 and Table 4.4).

For cultured cells, the level of expression of the particulate form did not vary between the parent cell lines, its variant metastatic sublines and the 'normal' cell line. Similarly, in the growing tumours, no alteration in the level of particulate expression was noted either between tumour variants or during tumour growth. However, the expression of the cytosolic and inactive forms of the enzyme varied between the sublines, both in cell culture and in growing tumours. The level of expression of cytosolic transglutaminase decreased with increasing metastatic potential, (except for the aberrant Met F line when grown in culture), with the level in the cultured 'normal' cell line being approximately twice that found in the weakest metastatic line, Met D, when grown in culture. Expression of the inactive form increased as cytosolic expression decreased, with no inactive antigen detectable in the cultured 'normal' cell line. As with activity, expression of all antigenic forms of transglutaminase was significantly lower in cultured cells than in growing tumours, with the

Table 4.3 : Levels of the three forms of transglutaminase antigen in HSV-2 induced hamster fibrosarcomas during the growth of the solid tumour.

Non-necrotic tissue was removed from tumours at the time points shown. Tissue. 200mg. was homogenised as in section 2.4.1 to give a 20% (w/v) homogenate and separated into cytosolic and particulate fractions by centrifugation (section 2.4.3). The 71.000g pellet was then twice extracted with non-ionic detergent (Lubrol-PX). as in section 2.4.2. and the two resultant PFS's combined with the cytosolic fraction. After dialysis for 1h against two changes of 5mM Tris-HCl pH7.4. 2mM EDTA. 2mM DTT (21). the proteins in the combined supernatants were separated by anion-exchange chromatography (~5mg protein loaded) (section 2.8.1.1) and the fractions assayed for transglutaminase antigen by the poly-l-lysine ELISA (section 2.6.3.2). Pooled antigen peaks were then quantitated for transglutaminase antigen by the sandwich ELISA given in section 2.6.3.1.

Data represents the mean of 6 experiments ± SE.

Variant		Transglutaminase antigen ng/ug DNA			
		Day 20	Day 24	Day 28	Day 34
Met D (1/20)	Particulate	1.67 ±0.31			1.89 ±0.25
	Inactive	1.76 ±0.22			2.03 ±0.47
	Cytosolic	7.42 ±0.36			7.17 ±0.29
Parent (6/19)	Particulate	2.41 ±0.21	2.36 ±0.37	2.32 ±0.13	2.04 ±0.19
	Inactive	2.48 ±0.26	2.27 ±0.62	2.42 ±0.25	2.73 ±0.30
	Cytosolic	6.12 ±0.23	5.84 ±0.37	5.59 ±0.42	5.90 ±0.41
Met B (20/20)	Particulate	2.44 ±0.20		2.63 +0.18	2.81 ±0.23
	Inactive	3.63 ±0.38		5.82 +0.31	6.84 ±0.22
	Cytosolic	5.71 ±0.14		3.63 +0.16	2.53 ±0.13
Met E (19/19)	Particulate	2.69 ±0.24			3.08 ±0.36
	Inactive	3.44 ±0.19			7.23 ±0.38
	Cytosolic	6.01 ±0.32			2.37 ±0.25

Table 4.4 : Levels of the three forms of transglutaminase antigen in cultured cells.

The three forms of transglutaminase antigen were extracted from cells and separated from one another as detailed in the legend to Fig. 4.5. Pooled antigen peaks were quantitated for transglutaminase antigen by the sandwich ELISA given in section 2.6.3.1.

Metastatic potential is given in parentheses.

Cell line		Transglutaminase antigen.		
		ng/10^6cells	ng/ug DNA	ng/mg protein
BHK	particulate	3.97±1.4	1.8±0.5	10.81±2.3
	inactive	ND	ND	ND
	cytosolic	13.96±2.6	6.3±1.7	38.07±5.8
Met D 1/20	particulate	4.14±1.6	1.5±0.7	10.66±1.8
	inactive	1.69±0.5	0.6±0.2	4.36±0.9
	cytosolic	7.85±2.0	2.7±1.8	20.24±3.6
Met C 4/18	particulate	4.20±1.3	1.5±0.4	10.29±1.4
	inactive	2.12±0.6	0.7±0.2	5.15±0.9
	cytosolic	7.52±1.3	2.7±0.4	18.38±3.2
Parent 6/19	particulate	4.95±1.4	1.7±0.3	11.81±2.1
	inactive	3.25±1.0	1.0±0.2	7.76±1.3
	cytosolic	6.24±1.8	2.1±1.0	14.89±3.7
Met B 20/20	particulate	6.15±1.4	2.8±0.9	16.05±2.9
	inactive	6.15±1.7	2.8+1.0	16.05±3.2
	cytosolic	3.39±0.8	1.5±0.3	8.85±1.3
Met E 19/19	particulate	5.91±1.9	2.7±1.0	14.18±2.3
	inactive	5.63±1.5	2.5±0.7	13.46±2.0
	cytosolic	3.14±0.7	1.4±0.5	7.45±1.7
Met F 20/20	particulate	3.85±1.2	1.7±0.9	9.35±1.8
	inactive	2.64±0.6	1.1±0.2	6.33±0.9
	cytosolic	7.50±2.1	3.3±1.0	18.27±3.4

aberrant Met F only conforming to the general trend (increasing metastatic potential, decreasing cytosolic transglutaminase and increasing inactive transglutamimase) when under the influence of the host environment. During tumour growth, cytosolic transglutaminase antigen levels in the highly metastatic variants continued to decrease, with a concommitent increase in expressed inactive antigen, the coefficient of correlation for this being $r = -0.99$. In the poorly metastatic variants (Met D and parent), no significant alteration in expression occurred in any of the antigenic forms during tumour growth.

4.5 DISCUSSION.

In order to quantitate the amount of cytosolic transglutaminase protein present in the various hamster fibrosarcoma lines it was necessary to develop a simple assay that did not require the full purification of protein from each individual tumour. Whilst developing such an assay - a sandwich ELISA for antigenic protein quantitation - it was observed that the affinity of two antibodies, raised against cytosolic transglutaminase from guinea-pig liver and rat liver, for cytosolic transglutaminase from hamster fibrosarcomas was equivalent to that for cytosolic transglutaminase from guinea-pig liver. This suggests that cytosolic tissue transglutaminase is a highly conserved protein, and may therefore be of great importance to normal cellular function.

Preliminary investigations undertaken to establish a cause for the reduction in cytosolic activity found with both increasing metastatic potential and during the growth of highly metastatic tumours, suggested that an inactivation of the cytosolic enzyme was occurring. Data supporting this hypothesis came from the measurement of transglutaminase antigen in the cytosolic supernatant from tumour homogenates, which indicated that while the activity of the cytosolic enzyme was at variance, depending on the metastatic potential of the tumour and its stage of growth, the level of transglutaminase antigen remained constant.

In order to extend these preliminary investigations, it was first of all important to establish to what extent the anti-cytosolic transglutaminase antibodies cross-reacted with other forms of transglutaminase. From these results it was apparent that the

antibodies were not specific for the cytosolic form against which they were raised since cross-reaction with the particulate form occurred, but they were specific for tissue transglutaminase, as no cross-reaction with the plasma transglutaminase, Factor XIII, was observed. The detection of antigenic particulate enzyme suggests that the two forms of tissue transglutaminase are not immunologically distinct as previously indicated (Chang and Chung, 1986)), but instead must share some common epitopes. This discrepancy can be explained by the fact that the previously published data relied on an Ochterlony diffusion method, which is less sensitive than the ELISA employed here.

The cross-reactivity of the antibodies with the particulate form of the enzyme enabled the distribution of transglutaminase expression to be ascertained. Each metastatic subline assayed, whether grown in cell culture or as a solid tumour, contained an inactive antigenic form of transglutaminase, separable from both the active forms by anion-exchange chromatography, which was not detectable in 'normal' cultured fibroblasts. The level of this inactive antigen was inversely related to the level of cytosolic antigen (and cytosolic activity) present. This observation was clearly demonstrated during the growth of the highly metastatic tumours, Met B, E and F with the level of the inactive form increasing at the expense of the active cytosolic form. However, in the weakly metastatic parent and Met D tumours, the level of inactive antigenic transglutaminase was low, and did not vary during tumour progression.

In contrast, in all the variants, whether examined in culture or during tumour growth, the levels of particulate transglutaminase antigen did not vary significantly, nor did the level of total transglutaminase protein.

The possibility of the appearance of an inactive transglutaminase antigen being the major cause of the altered cytosolic activity, by a perturbation in the expression of the active cytosolic form, cannot be ruled out at this stage. This theory is strengthened by the apparent relationship between the inactive antigen and the cytosolic form of transglutaminase, and the observation that no inactive antigen is detectable in 'normal' fibroblasts in culture.

It was now important to demonstrate the presence of this inactive protein in other malignant neoplasms where reduced transglutaminase activity has been observed, and further to confirm its absence in normal tissue. To this end, the following chapter looks at the level and distribution of transglutaminase activity and antigen in four rat sarcomas of differing metastatic potential and in normal rat and hamster liver.

5: DISTRIBUTION OF TRANSGLUTAMINASE ACTIVITY AND ANTIGEN IN NORMAL LIVER AND NON-METASTASISING AND METASTASISING RAT SARCOMAS.

5.1 Introduction.

Previous work undertaken in this laboratory has indicated that total transglutaminase activity decreases with tumour growth in two metastatic rat sarcoma lines whilst in two non-metastatic rat sarcoma lines total activity remained unchanged (Barnes et al, 1984, 1985; Hand et al, 1987). It has also been observed that as transglutaminase activity decreases during the growth of the P8 tumour (highly metastatic), the levels of unbound spermidine and putrescine (two polyamine substrates of transglutaminase) increase, possibly reflecting alterations in the levels of transglutaminase activity (Hand et al, 1987).

Another model system, comprising metastatic and non-metastatic tumours, in a different species, was therefore available that was known to show decreases in total transglutaminase activity in a manner similar to those seen in the hamster model. Analysis of the distribution of active and antigenic transglutaminase might therefore confirm the existence of the inactive form of transglutaminase in another species.

Further, to confirm the inactive transglutaminase as a novel protein associated with carcinomas, and not normal tissue, activity and antigen distributions were also assessed in normal liver tissue. This tissue has been used by other workers either as a source of standard transglutaminase or as a control tissue (Barnes et al, 1985; Hand et al, 1987). Its use therefore provides an 'in vivo' control for the absence of inactive transglutaminase in normal tissue, and also allows comparisons to be made between the activity results of other workers and those obtained in this study.

5.1.1 Description of rat sarcomas.

The origins and growth characteristics of the four rat sarcomas used in this study have been described by Moore, 1972, and details are given in section 2.3.1. To recap :-

P7 : osteosarcoma induced by intraperitoneal injection of ^{32}P.

P8 : fibrosarcoma induced by intraperitoneal injection of ^{32}P.

MC3 : fibrosarcoma induced by methylcholanthrene dose.

CC5 : osteosarcoma induced by cupric-chelated-N-hydroxy-2-acetyl-amino-fluorene dose.

Assessment of metastatic potential, by the detection of lung metastases using Indian ink (section 2.3.5) exclusion has indicated that the osteosarcoma CC5 and the fibrosarcoma MC3 are non-metastatic; the osteosarcoma P7 metastasises occassionally, and the fibrosarcoma P8 metastasises frequently. Assessment of the metastatic potential of P7 and P8 by resection of the primary load (section 2.3.4) and measurement of the number of animals showing lung metastases indicated the metastatic potential of P7 to be 2/8, and P8 to be 7/8.

5.2 Transglutaminase measurement and distribution in normal and neoplastic tissue.

To ascertain whether or not the inactive form of the enzyme protein found in the hamster fibrosarcoma was present in other malignant tumours, in particular those occurring in other animal species, and in normal tissue, its presence was investigated in four rat sarcomas and normal liver from rat and hamster. Tissue was extracted with non-ionic detergent (Lubrol-PX) and the different forms of transglutaminase separated by anion-exchange chromatography. Eluent fractions were assayed for transglutaminase activity and transglutaminase antigen. Pooled activity peaks were then assayed more accurately using ^{14}C-putrescine incorporation into N,N'-dimethylcasein and pooled antigen peaks quantitated for transglutaminase protein using a sandwich ELISA.

5.2.1 In liver, as a normal tissue.

As expected, the activity profile for rat liver (Fig. 5.1) was similar to that obtained by previous workers (Chang and Chung, 1986; Hand et al, 1987), indicating two forms of transglutaminase, the particulate eluting at 0.15 - 0.25 M NaCl and the cytosolic eluting at 0.35 - 0.45 M NaCl. Activity profiles obtained from hamster liver were comparable to those obtained from rat liver (Fig.5.1). The antigen profiles for rat and hamster liver (Fig. 5.1) gave similar patterns to those obtained for activity, with only two antigen peaks detected. These antigen peaks corresponded to the activity peaks of the

particulate and cytosolic forms. There was no evidence to indicate the presence, in either rat or hamster liver, of an inactive antigenic form of transglutamianse.

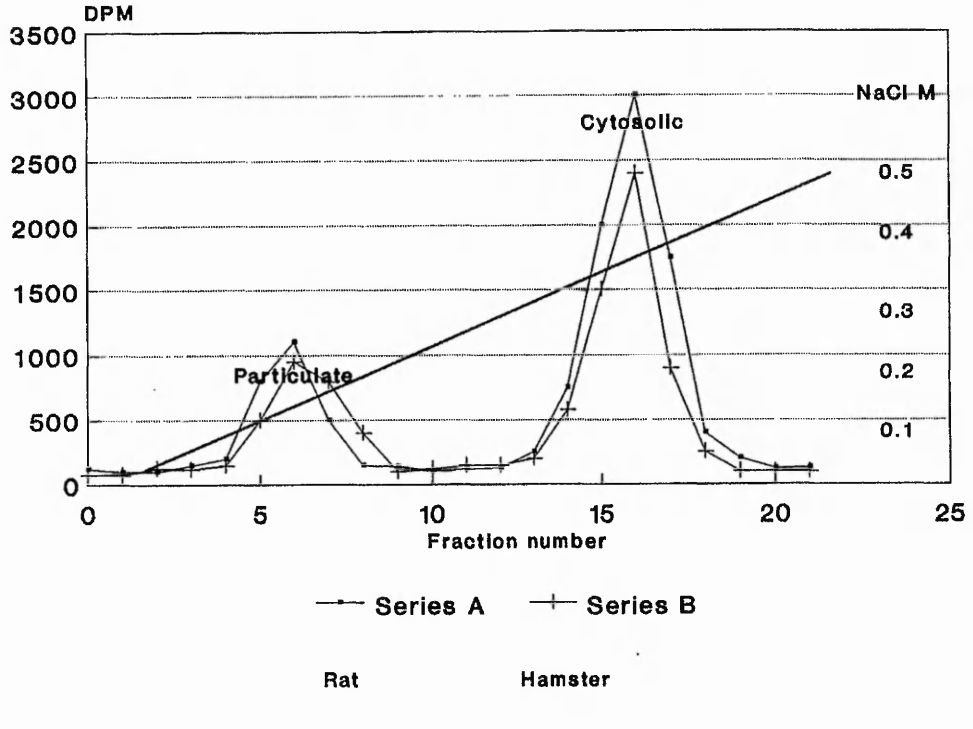
The levels of total activity, cytosolic activity and particulate activity (Table 5.1) found in rat liver were within the range of activities observed by other workers (Chang and Chung, 1986; Barnes et al, 1985; Hand et al, 1989) for this tissue. The level of cytosolic transglutaminase activity was approximately 4 times that of the particulate form, again within the expected range. No significant difference was observed between the levels of activity and antigen found in livers from either of the two species.

Fig. 5.1 : Typical elution profiles from anion-exchange chromatography of liver extracts.

Tissue (1g) was homogenised to give a final 20% (w/v) homogenate in homogenising buffer containing 1% (w/v) Lubrol-PX. Protein (approximately 10mg, equivalent to approximately 8ug total transglutaminase antigen) was loaded onto a Mono-Q column and eluted with a gradient of 0 - 0.5 M NaCl according to the procedures outlined in section 2.8.1.1.

a) Fractions, 500ul, were collected, 25ul aliquots removed and diluted 1:1 with PBS before being assayed for antigenic transglutaminase by the poly-l-lysine ELISA (section 2.6.3.2) to give antigen elution profiles.

b) Fractions were also assayed for transglutaminase activity by the ^{3}H-putrescine assay (section 2.6.1.2) to give activity elution profiles.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2500
2000
1500
1000
500
0
NaCl M
0.5
0.4
0.3
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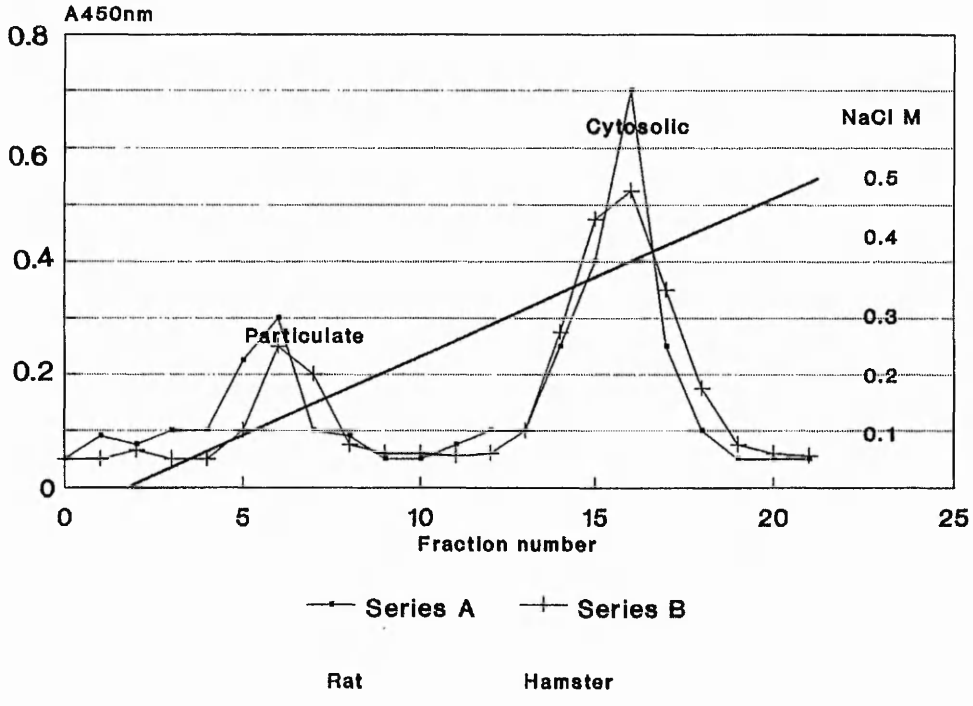
Cytosolic
Particulate
0
5
10
15
20
25
Fraction number
Series A
Series B
Rat
Hamster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0.4
0.2
0
NaCl M
0.5
0.4
0.3
0.2
0.1
Cytosolic
Particulate
0
5
10
15
20
25
Fraction number
Series A
Series B
Rat
Hamster

5.2.2 In two non-metastasising rat sarcomas, MC3 and CC5.

The two tumour lines, MC3 and CC5, have been designated as 'non-metastatic' sarcomas. No metastatic deposits have been observed from tumour growths of either of these two lines, up to 60 days growth.

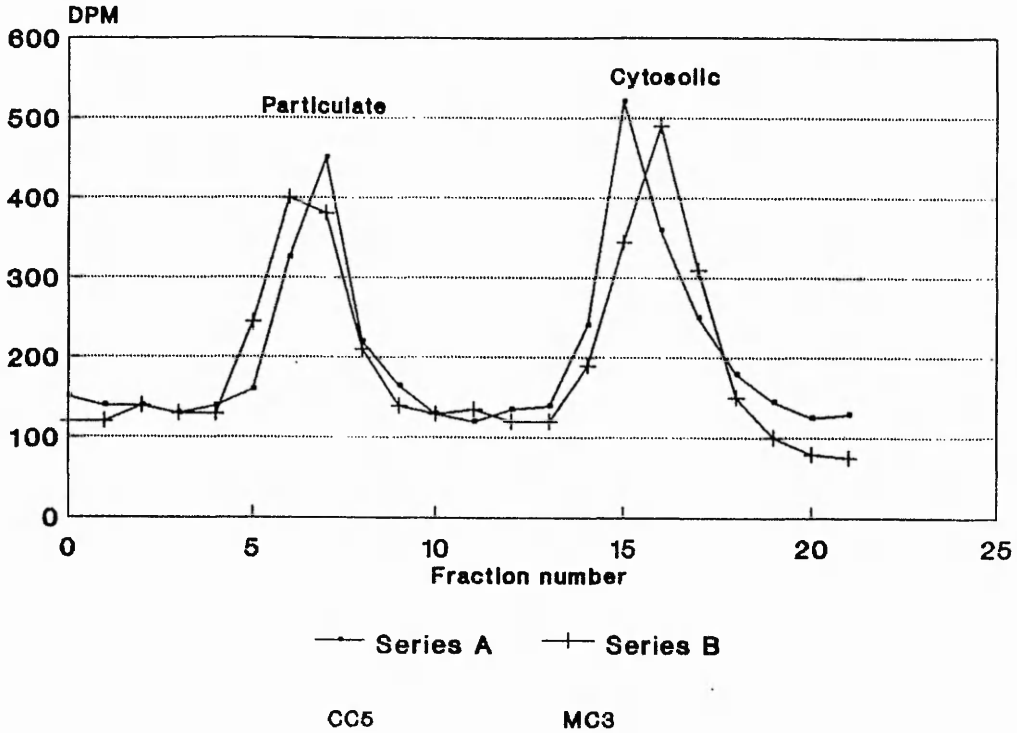
From activity and antigen profiles, it was apparent that at both late (day 50) and early (day 15) stages of growth, only the two active forms (the particulate and cytosolic) of the tissue enzyme were present (Fig.5.2a and b) in these two tumour lines. However, in both MC3 and CC5 there was a small decrease (approximately 10%) in the amount of cytosolic activity present during tumour growth (Table 5.1). This decrease in cytosolic activity was not accompanied by any significant decrease in cytosolic antigen (Table 5.1), thus, as with the hamster sarcomas, a small decrease in the specific activity of the cytosolic enzyme appears to accompany tumour growth. No significant alteration in the amount of particulate activity or antigen was observed during growth in either of the two tumour lines (Table 5.1), this again is similar to observations made during the growth of the hamster sarcomas.

Fig. 5.2 : Typical elution profiles from anion-exchange chromatography of extracts of the non-metastasising rat sarcomas CC5 and MC3.

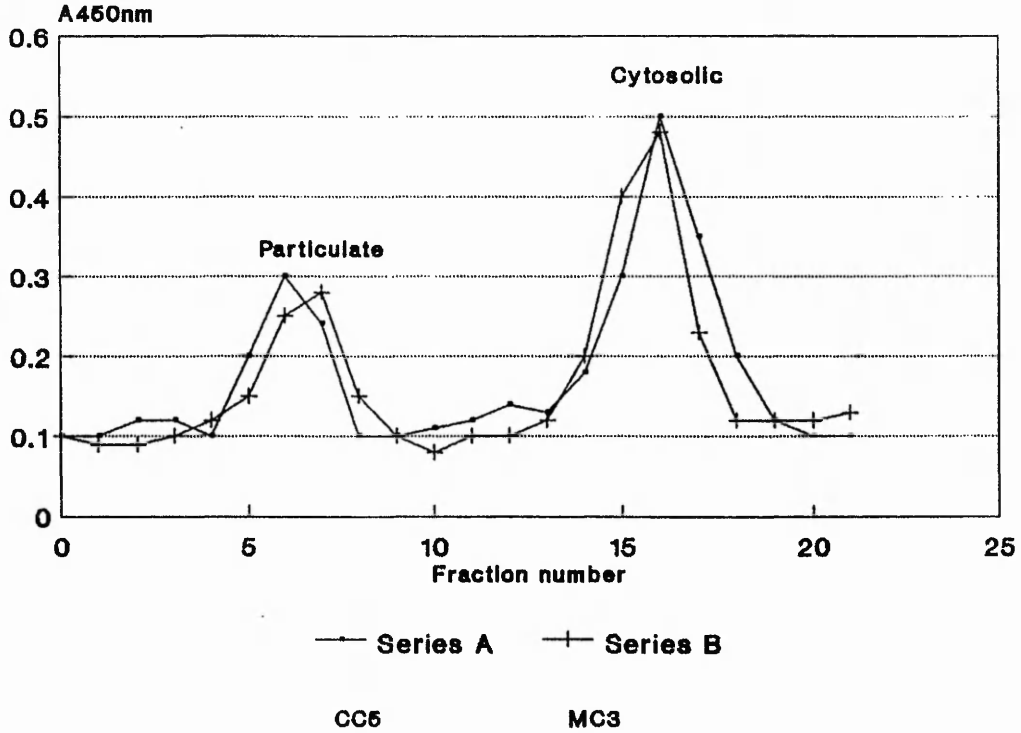
Tissue (500mg) was homogenised to give a final 20% (w/v) homogenate in homogenising buffer containing Lubrol-PX (1% (w/v)). Protein (approximately 10mg, equivalent to approximately 600ng total transglutaminase antigen) was loaded onto a Mono-Q column and eluted as described in the legend to Fig.5.1.

a) Fractions, 500ul, were collected and assayed for antigenic transglutaminase by the poly-l-lysine ELISA (section 2.6.3.2) to give antigen elution profiles.

b) Fractions were also assayed for transglutaminase activity by the ^{3}H-putrescine assay (section 2.6.1.2) to give activity elution profiles.



CC5 MC3



CC5 MC3

5.2.3 In two metastasising rat sarcomas, P7 and P8.

To test whether the inactive form of the tissue enzyme found in the hamster fibrosarcomas was present in malignant tumours of another species its presence was investigated in the highly metastatic rat fibrosarcoma P8, and the weakly metastatic rat osteosarcoma P7.

Activity profiles, for early (day 15) and late (day 32) stages of growth of both P7 and P8, again indicated the presence of two forms, particulate and cytosolic. Antigen profiles, however, revealed the presence of a third antigenic peak corresponding in elution to the inactive form found in hamster fibrosarcomas (Fig 5.3a and b).

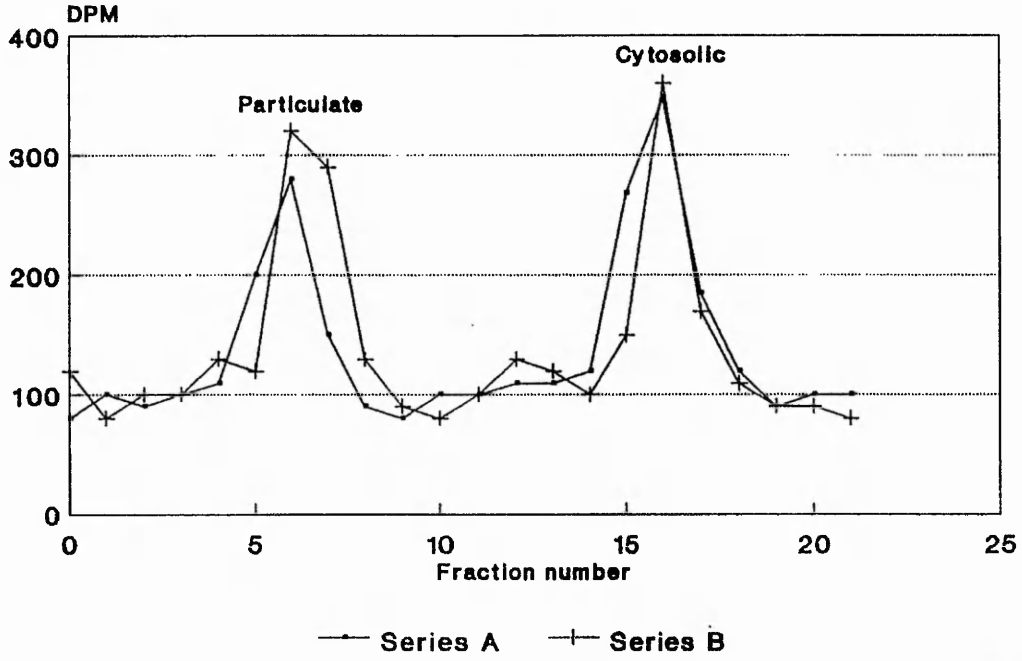
As with MC3 and CC5, cytosolic transglutaminase activity decreased during the growth of the P7 and P8 tumours. In the highly metastatic P8 tumours, this decrease led to the loss of approximately 70% of the initial cytosolic activity by day 32 of growth; whilst in the weakly metastatic P7 tumours, a less dramatic, but still significant, loss of 30% of the initial activity occured over the same time period (Table 5.1). Accompanying the decrease in cytosolic activity seen in both tumour lines, was a decrease in cytosolic antigen expression (Table 5.1), which accounted for approximately 65% of the lost cytosolic activity. Again, not all of the loss of cytosolic activity could be accounted for by a loss of cytosolic antigen, a decrease in the specific activity of the enzyme also occured. As expression of the cytosolic antigen decreased, the expression of the inactive antigen increased (Table 5.1), maintaining their combined levels such that the loss in expressed cytosolic antigen was compensated for by increased expression of the inactive antigen.

Fig. 5.3 : Typical elution profiles from anion-exchange chromatography of extracts from the metastasising rat sarcomas, P7 and P8.

Tissue (500mg) was homogenised to give a final 20% (w/v) homogenate in homogenising buffer containing 1% (w/v) Lubrol-PX. Protein (approximately 10mg, equivalent to approximately 400ng total transglutaminase antigen) was loaded onto a Mono-Q column and eluted as in the legend to Fig.5.1.

a) Fractions, 500ul, were collected and assayed for antigenic transglutaminase by the poly-l-lysine ELISA (section 2.6.3.2) to give antigen elution profiles.

b) Fractions were also assayed for transglutaminase activity by the ^{3}H-putrescine assay (section 2.6.1.2) to give activity elution profiles.


DPM
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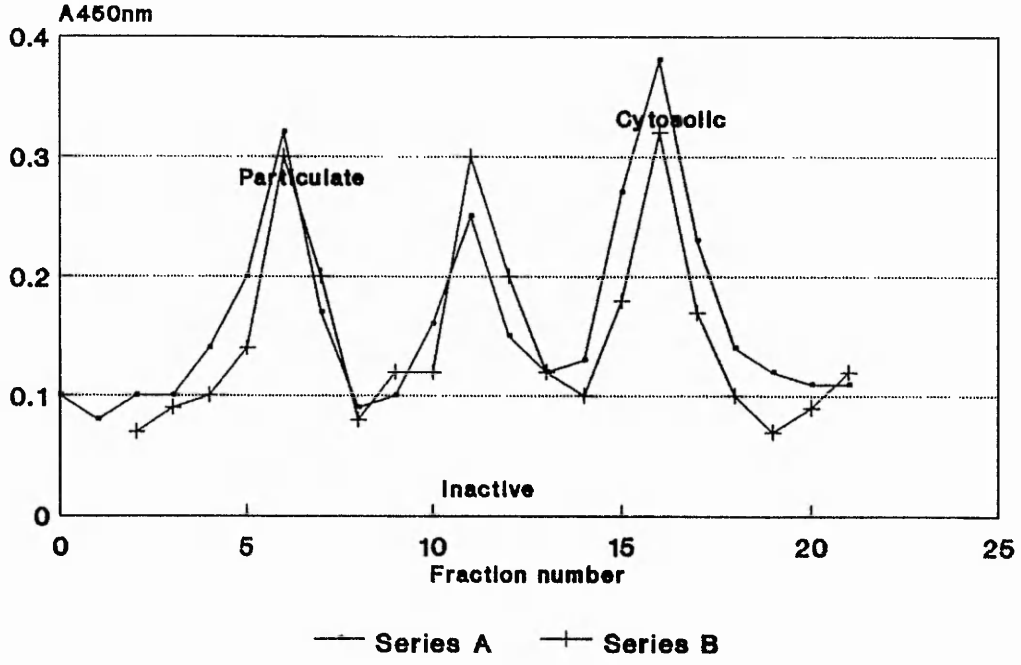
300
200
100
0
Particulate
Cytosolic
0
5
10
15
20
25
Fraction number
Series A
Series B

P7 P8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0.3
0.2
0.1
0
Particulate
Cytosolic
Inactive
0
5
10
15
20
25
Fraction number
Series A
Series B

P7 P8

Table 5.1 : The distribution of tissue transglutaminase in normal liver, two non-metastatic rat sarcomas (CC5 and MC3) and two metastatic rat sarcomas (P7 and P8).

The tissue transglutaminases were extracted from 20% (w/v) tissue homogenates with buffer containing Lubrol-PX (section 2.4.2), separated by anion-exchange chromatography (Mono-Q, section 2.8.1.2) and the levels of active and antigenic transglutaminase in each fraction were measured as described in the legends to Figs. 5.1, 5.2 and 5.3. Activity in pooled activity peaks was measured by the Ca^{2+} dependent incorporation of ^{14}C-putrescine into N,N'-dimethylcasein (section 2.6.1.1). Antigen concentration in pooled antigenic peaks was quantitated by sandwich ELISA (section 2.6.3.1).

d denotes the number of days of tumour growth.

ND denotes 'not detectable'.

The suffix R indicates rat as the species of origin and the suffix H indicates hamster.

Data represents the mean of 6 experiments ± SE.

Tissue	Transglutaminase activity U/mg DNA		Transglutaminase antigen ng/ug DNA		
	Particulate	Cytosolic	Particulate	Inactive	Cytosolic
$Liver_R$	1278 ± 96	5327 ± 112	20.7 ± 2.5	ND	66.8 ± 5.7
$Liver_H$	1385 ± 119	5183 ± 84	21.6 ± 1.5	ND	59.2 ± 4.9
CC5 d15	103 ± 23	207 ± 16	1.3 ± 0.5	ND	2.2 ± 0.8
CC5 d32	85 ± 17	179 ± 12	1.2 ± 0.3	ND	2.0 ± 0.6
CC5 d50	93 ± 31	167 ± 15	1.6 ± 0.4	ND	1.9 ± 0.3
MC3 d15	74 ± 9	186 ± 11	0.9 ± 0.3	ND	2.0 ± 0.6
MC3 d32	87 ± 13	165 ± 8	1.1 ± 0.7	ND	1.9 ± 0.4
MC3 d50	90 ± 20	154 ± 9	1.1 ± 0.4	ND	2.0 ± 0.3
P7 d15	142 ± 31	218 ± 26	1.7 ± 0.6	0.5±0.1	3.0 ± 0.3
P7 d32	115 ± 24	144 ± 17	1.6 ± 0.3	1.1±0.3	2.1 ± 0.4
P8 d15	91 ± 22	200 ± 15	1.1 ± 0.1	3.0±0.2	2.1 ± 0.2
P8 d32	80 ± 17	60 ± 11	1.2 ± 0.2	4.3±0.2	1.2 ± 0.3

5.3 Transglutaminase measurement and distribution in secondary tumours of P7 and P8.

Secondary tumours, or 'metastases', are derived from single cells, or small clumps of cells, that have successfully undergone the neccesary steps involved in dissemination. These steps include :-

detachment of the cell from the primary tumour and subsequent infiltration into the adjacent tissue;

intravasation (migration of cells into the circulation or lymphatics);

extravasation (migration of cells from the circulation or lmyphatics);

migration through perivascular stroma;

clonal growth in parenchyma of invaded organ.

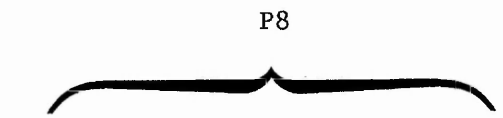
It may be expected that at an early time point in the growth of a secondary tumour, it will to a great extent be made up of cells cloned from, and therefore identical to, the original metastatic cell or cells. Thus to evaluate the characteristics of the 'metastatic' cell (ie. a cell capable of undergoing all the above steps) it is feasible to look at an early stage of the secondary tumour.

5.3.1 Origination of secondary tumours.

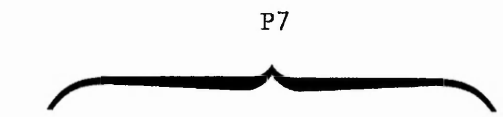
Tumours from the two metastatic rat sarcomas P7 and P8, passaged at day 21, were resected at day 12 of their growth. Animals were sacrificed by cervical dislocation 60 days after initiation of primary tumour growth (48 days post-resection). Secondary growths, of

an average diameter of 8mm. were removed from the lungs and assayed both for transglutaminase activity and antigen. Samples of tissue from each 'metastases' were frozen. and put into storage. at -120°C for future development of metastatic variants.

Lineage of secondary tumours :-



P8M1 P8M2 P8M3 P8M4 (metastases)



P7M1 P7M2 P7M3 P7M4 (metastases)

5.3.2 Measurement and distribution in P8M 1 - 4. P7M 1 - 4 and their parent tumours.

Resected primary tumours were homogenised. and the transglutaminases that they contained were extracted. separated. assayed and quantitated as in section 5.2.

Secondary growths were removed. from the lungs of animals whose primary tumour load had been resected. and the surrounding lung tissue dissected away. These tumours were then treated in an identical fashion to the primary tumours.

Activity profiles indicated the presence of the two active forms of the enzyme. the particulate and the cytosolic in all tumours whether primary or secondary. There was a significant difference

between the levels of cytosolic transglutamianse activity in the primary and secondary tumours of both P8 and P7 origin (Table 5.2). In the tumours P8M 1 - 4, derived from the highly metastatic P8 tumour line, cytosolic transglutaminase activity was 30 - 40 % lower than in the primary tumour (at day 12 of growth). Secondaries derived from the weakly metastatic P7 tumour line (P7M 1 - 4) contained less than 40% of the cytosolic activity found in the primary tumours (at day 12 of growth). In all tumours, whether primary or secondary, particulate activity remained constant, ranging from 100 - 150 U/mg DNA.

Profiles showing the elution of antigenic transglutaminase from an anion-exchange column (Mono-Q) indicated the presence of three antigenic forms in all tumours. By comparing the levels of expression of each form in the different tumours, it was apparent that the reduced cytosolic activity seen in the metastases of both P8 and P7 was due to a decrease in the expression of the cytosolic form of the enzyme, with no significant alteration to its specific activity (Table 5.2). This decrease in the expression of the cytosolic enzyme was mirrored by an increased expression of the inactive antigenic form (Table 5.2). As with activity, particulate expression was not significantly altered (Table 5.2).

Table 5.2 : The distribution of tissue transglutaminase in primary and secondary tumours of the tumour lines P8 and P7.

The tissue transglutaminases were extracted from 20% (w/v) tissue homogenates (500mg wet weight primary tumour; ~100mg wet weight secondary tumour) with Lubrol-PX (section 2.4.2) and separated by anion-exchange chromatography (1ml Mono-Q, section 2.8.1.2). Transglutaminase activity was measured by the Ca^{2+} dependent incorporation of ^{14}C-putrescine into N,N'-dimethylcasein (section 2.6.1.1), and antigen levels were quantitated by sandwich ELISA (section 2.6.3.1).

d denotes the number of days of tumour growth.

Tissue	Transglutaminase activity U/mg DNA		Transglutaminase antigen ng/ug DNA		
	Particulate	Cytosolic	Particulate	Inactive	Cytosolic
P8 d12	121	213	1.1	3.1	2.4
P8M 1	126	128	0.9	3.9	1.5
P8M 2	137	165	1.2	3.5	1.7
P8M 3	109	142	0.9	4.1	1.2
P8M 4	113	118	1.0	4.0	0.8
Average	121 ± 11	138 ± 17	1.0 ± 0.1	3.9±0.2	1.3 ± 0.3
P7 d12	151	245	1.6	0.5	3.4
P7M 1	129	102	1.5	2.9	1.2
P7M 2	134	87	1.5	3.2	0.7
P7M 3	142	113	1.7	2.8	0.9
P7M 4	123	124	1.4	2.4	1.6
Average	132 ± 7	107 ± 14	1.5 ± 0.1	2.8±0.3	1.1 ± 0.4

5.4 Discussion.

In Chapter 3, data was presented indicating that the level of cytosolic transglutaminase activity and the metastatic potential of a tumour were inversely related. This was followed up in Chapter 4 with data that explained the decrease in cytosolic activity as being the result of reduced expression of the cytosolic form. This reduction in cytosolic transglutaminase expression was explained by the appearance of an inactive form of transglutaminase whose expression correlated inversely with the level of expression of the cytosolic transglutaminase.

In order to extend these preliminary investigations, undertaken on one tumour type from one species, it was important to demonstrate two things. Firstly, the absence, or presence, of this inactive form of transglutaminase protein in normal tissue, in order to establish the protein as either an abnormality produced solely by malignant cells, or a normal inactive stage of the synthesis of active transglutaminase. Secondly, the existence of an inverse relationship between cytosolic transglutaminase activity and metastatic potential in other tumour systems, involving the appearence of the inactive enzyme form.

Studies with normal liver, from both rat and hamster, indicated that this tissue contained only two detectable forms of antigenic transglutaminase, corresponding to the active cytosolic and particulate forms of the enzyme. No inactive form of transglutaminase was observed. This suggests that either the processing of the inactive form in normal liver is so rapid as to render it undetectable by the present assay, or that its existence is due to a malfunctioning in the

synthetic pathway of transglutaminase, either pre- or post-transcriptional, that occurs with the onset of malignancy.

Previous work has shown that alterations in the level of total transglutaminase activity occur during the growth and progression of the two metastasising rat sarcomas P8 and P7 (Barnes et al 1985; Hand et al 1988) but not during the growth of the two non-metastatic rat sarcomas MC3 and CC5 (Barnes et al, 1985). Investigations undertaken in the course of this study, confirm the reductions in activity found during the growth of P7 and P8, and further they indicate that these reductions are caused by a loss of cytosolic transglutaminase activity. In contrast to earlier investigations, a slight decrease in activity was also observed during CC5 and MC3 tumour growth, and again this was attributable to a loss in cytosolic activity.

To investigate the cause of reductions in cytosolic transglutaminase activity in these rat sarcomas, transglutaminase expression during tumour growth was also monitored. In the two non-metastasising tumour lines (MC3 and CC5), only the cytosolic and particulate forms of antigenic transglutaminase were detectable. No significant decrease in the expression of the cytosolic form of tissue transglutaminase occurred between day 15 and day 50, thus indicating a slight decrease in the specific activity of this enzyme form during tumour growth. As with the hamster fibrosarcomas, three antigenic transglutaminases were present in the metastasising P8 and P7 tumour lines, the particulate, the cytosolic and the inactive form. These two metastastic tumour lines also contained significantly lower levels of cytosolic enzyme protein on day 32 of growth, compared to the levels present at day 15. This reduction in expression was greatest in the

highly metastatic P8 tumour line, as was the observed decrease in activity. However, in neither of these two lines did the reduced expression fully account for the loss in activity. In P7, approximately 80% of the lost activity could be accounted for by decreased enzyme expression and in P8 only 60%, therefore indicating again that the specific activity of the cytosolic form was decreasing with tumour growth. Mirroring the decrease in expressed cytosolic protein was an increase in inactive protein, confirming the inverse relationship between the two forms that was apparent in the hamster fibrosarcoma system. This was most clearly demonstrated during the growth of the highly metastatic rat fibrosarcoma, P8, with the level of the inactive form increasing at the expense of the active cytosolic form.

In contrast to the differences in expression and activity of the cytosolic enzyme found in the four tumour lines during growth, neither the expression nor the activity of the particulate form of tissue transglutaminase varied significantly during tumour growth.

These findings suggest that the presence of the inactive transglutaminase antigen may be common to a number of malignant neoplasms; decreased levels of transglutaminase activity have already been observed in other tumour systems (Birckbichler et al, 1976, 1977; Delcros et al, 1986, 1987; Hand et al, 1987; Romijn et al, 1989) and the inactive antigen may be involved in these. The inability to detect the inactive antigen in non-metastatic tumours suggests that there is the potential to use tissue transglutaminase, and specifically the inactive antigen, as a marker for metastatic potential in tumour diagnosis.

Further evidence to link the production of the inactive enzyme form with the metastatic phenotype came from investigations

carried out on the secondary growths of the P7 and P8 tumour lines. All the secondary growths examined contained significantly higher levels of inactive antigen than their parent tumours, with the concomitant decrease in cytosolic activity. Clonal growth of a metastatic cell in the formation of a secondary tumour growth, could lead to either an increase or decrease, over normal levels, of any protein that that cell contained. Thus the concentration of any protein that is either necessary for metastases, or is advantageous to the metastatic cell, will tend to be higher in the secondary tumour than in the primary tumour. Conversely, any protein whose absence is of advantage to the metastatic cell will be found in lower concentrations within the secondary tumour as compared to the primary tumour. The levels of expressed inactive and cytosolic transglutaminase increase and decrease respectively from primary to secondary tumour. This suggests that the original metastatic cell/s may produce inactive antigen rather than active cytosolic enzyme, and that therefore the absence of the active form, or the presence of the inactive antigen, is advantageous to the metastatic cell.

6: CHARACTERISATION OF INACTIVE AND PARTICULATE TRANSGLUTAMINASE.

6.1 Introduction.

Separation of detergent extracts from rat and hamster sarcomas by anion-exchange chromatography has indicated the presence of three forms of transglutaminase antigen, an inactive form and the active particulate and cytosolic forms. All three antigenic forms of transglutaminase are clearly separable from one another by this method (refer to section 4.5.1). Unlike tumours, the presence of the inactive antigenic form could not be demonstrated in extracts from normal hamster fibroblasts, (section 4.4.4) and normal hamster or rat liver (section 5.2.1). Measurement of antigen levels in these sarcomas, during growth, indicated an inverse relationship between the expression of the inactive transglutaminase antigen and the level of cytosolic transglutaminase activity, suggesting that these two forms of the enzyme may be inter-related. There is also uncertainty at the present time as to the relationship of the active particulate and cytosolic forms of tissue transglutaminase, and whether they are in fact distinct and discrete enzymes.

This chapter aims to establish any similarities, and dissimilarities, both physical and immunological, that may exist between the three antigenic forms.

All elution profiles in this chapter were produced by assaying eluent fractions with either the ^{3}H-putrescine assay (section 2.6.1.2) or the poly-l-lysine ELISA (section 2.6.3.2), to give activity and antigen profiles respectively. Actual activities were calculated using the ^{14}C-putrescine assay (section 2.6.1.1) and antigen concentrations were calculated using the quantitative sandwich ELISA (section 2.6.3.1).

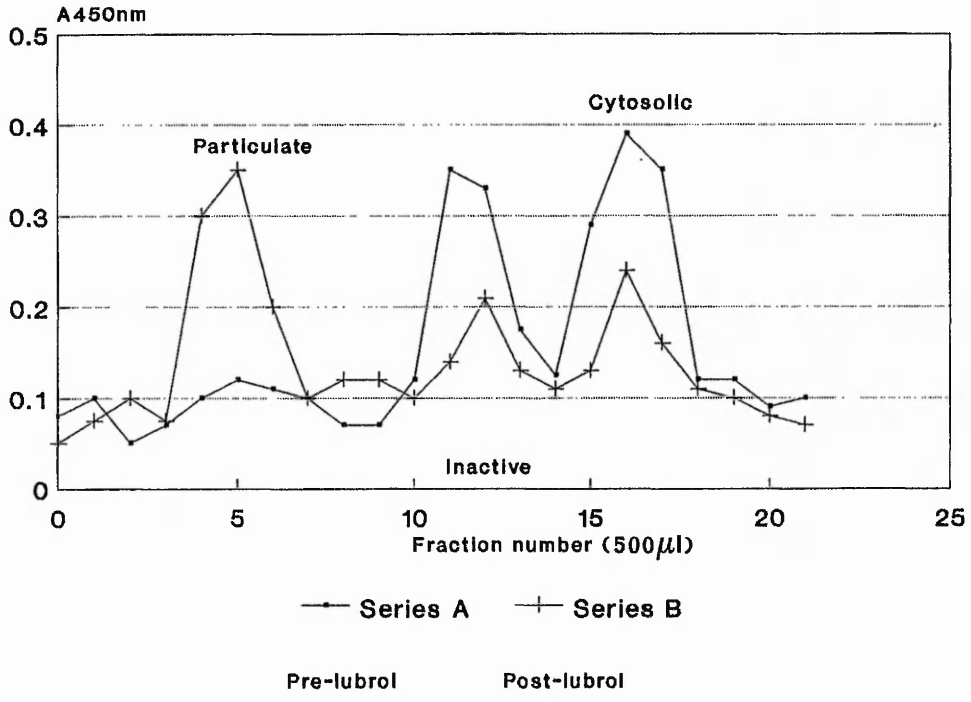
6.2. Comparison of the three forms of antigenic transglutaminase.

6.2.1 Subcellular localisation.

To elucidate the subcellular localisation of the inactive form, rat and hamster fibrosarcomas were homogenised in homogenising buffer (0.25M sucrose, 5mM Tris/HCl pH7.4, 2mM EDTA, 2mM DTT, 5mM Benzamidine, 1mM PMSF in 1% (v/v) DMSO) and the homogenate separated into cytosolic and particulate fractions by centrifugation at 71,000g for 45min. Transglutaminases present in the cytosolic fraction were then separated by anion-exchange chromatography and the eluent fractions assayed for transglutaminase antigen. Membrane associated transglutaminases were extracted from the 71,000g particulate fraction with homogenising buffer containing 1% (w/v) Lubrol-PX (section 2.4.2) and separated into a 71,000g pellet and supernatant (lubrol extract). This lubrol extract was then subjected to anion-exchange chromatography and the resultant eluent fractions were assayed for transglutaminase antigen. Comparison of the antigen profiles obtained from the cytosol and the lubrol extract indicated that the majority of the inactive antigen and the cytosolic antigen (70% and 80% respectively) were found in the initial cytosol fraction (Fig.6.1). Hence of the three antigenic forms of transglutaminase found in the fibrosarcoma, two are predominantly cytosolic while the third is membrane associated.

Figure 6.1 : Anion-exchange chromatography antigen elution profiles for P8 tissue, with, or without, Lubrol-PX extraction.

Non-necrotic tissue was removed from P8 tumours after 15 days growth, and homogenised in non-lubrol buffer to give a 20% (w/v) homogenate (section 2.4.1). The cytosolic fraction was removed by centrifugation at 71,000g for 45min (section 2.4.1) and the proteins (~5mg) separated by anion-exchange chromatography on a 1ml Mono-Q (Pharmacia) column (section 2.8.1.1). Eluent fractions were assayed for antigen by a poly-l-lysine ELISA. Proteins in the remaining 71,000g pellet were extracted with homogenising buffer containing 1% (w/v) Lubrol-PX (section 2.4.2) and separated into a particle free supernatant (PFS) by centrifugation at 71,000g for 45min. PFS proteins (~5mg) were then separated on a Mono-Q column as above, and the eluent fractions assayed for antigenic transglutaminase by a poly-l-lysine ELISA.


A450nm
0.5
0.4
0.3
0.2
0.1
0
Cytosolic
Particulate
Inactive
0
5
10
15
20
25
Fraction number (500μl)
Series A
Series B
Pre-lubrol
Post-lubrol

6.2.2 Molecular weight.

The molecular weight (Mr) of each of the three forms was estimated by size exclusion gel filtration chromatography using a TSK SW 3000 molecular seive column (section 2.8.2.1). Protein standards ranging from Mr 18,400 - 232,000 daltons (Table 6.1a) were used to construct a calibration plot of $\log_{10}$Mr against partition coefficient (Kav) for the column (Fig.6.2).

To assess and compare the molecular weights of the particulate, cytosolic and inactive antigens, tumour lubrol extracts (~5mg protein) were separated by anion-exchange chromatography and the relevant fractions pooled to give partially purified enzyme forms. These semi-pure enzyme preparations were concentrated by reverse dialysis using Sephadex G-25 (to give 20 - 80ug protein /ml), and then applied to the precalibrated (Fig.6.2) molecular seive column for assessment of their different molecular weights. The approximate Mr of the three antigenic forms was calculated from their elution profiles (Fig.6.3), and these gave approximate values of 80,000 daltons for the cytosolic enzyme, 100,000 daltons for the particulate and 120,000 daltons for the inactive transglutaminase antigen (Table 6.1b); the values for the particulate and cytosolic forms are comparable to those reported by Chang and Chung, 1986. Even when allowing for an estimated experimental error of ±2000 daltons based on the fraction size of 250ul, the values obtained for each of the three forms are significantly different from one another.

Figure 6.2 : Molecular weight calibration of a TSK 3000 SW gel filtration column for the estimation of the molecular weight of the transglutaminase forms.

An Ultropac TSK 3000 SW column (26.5ml) (Pharmacia-LKB) was calibrated with the protein molecular weight standards (1mg), as given in Table 6.1, according to the procedures laid out in section 2.8.2.1. Arrows indicate the mean elution positions of each form from rat and hamster.

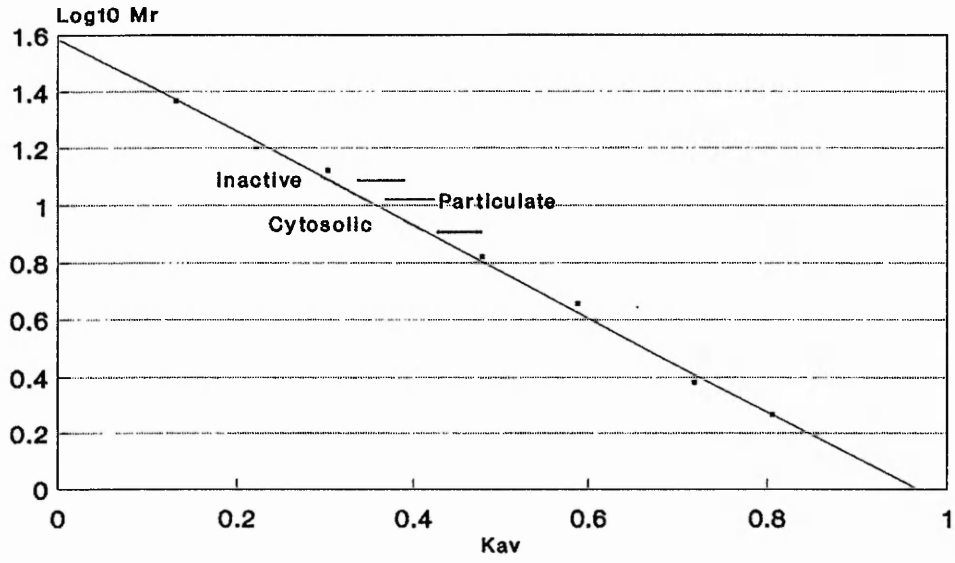

Log10 Mr
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
Kav
Inactive
Particulate
Cytosolic

Figure 6.3 : Typical antigen profiles of the elution of each form from a TSK 3000 SW gel filtration column.

Semi-purified samples (from anion-exchange) of each form of antigenic transglutaminase (~400ng), from the rat sarcoma P8, were applied to a TSK 3000 SW column (26.5ml) and eluted as described in section 2.8.2.1. Eluent fractions were assayed for antigenic transglutaminase by a poly-l-lysine ELISA (section 2.6.3.2). Molecular weights were estimated by reference to the calibration graph given in Figure 6.2.

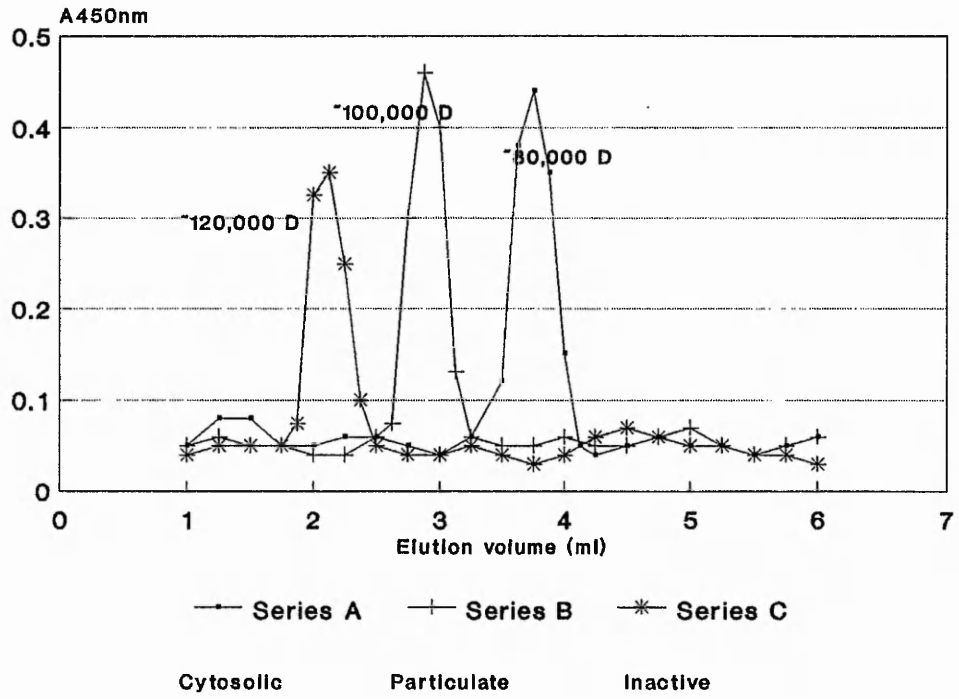

A450nm
0.5
0.4
0.3
0.2
0.1
0
~100,000 D
~80,000 D
~120,000 D
0
1
2
3
4
5
6
7
Elution volume (ml)
Series A
Series B
Series C
Cytosolic
Particulate
Inactive

Table 6.1 : Molecular weight determination of the three antigenic forms of transglutaminase from rat and hamster sarcomas.

Protein standards (200ul of a 5mg / ml solution) (Table 6.1a) and samples of the three tissue transglutaminase forms (5 - 15 ug protein) (semi-purified as described in the text) were chromatographed individually on a TSK SW 3000 molecular seive column as detailed in section 2.8.2.1. Kav was calculated from the formula :

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

where :- Ve is the elution volume (calculated by peak triangulation)

Vo is the void volume (7.2ml, determined using blue dextran)

Vt is the total column volume (26.5ml).

The estimated molecular weights shown in Table 6.1b were extrapolated from the calibration plot shown in Fig.6.2, and are presented as the mean of three chromatograms ± SE.

a : standard proteins.

Protein	Molecular weight	Kav
Aldolase	158,000	0.2205
Bovine serum albumin (dimeric)	132,000	0.3026
Bovine serum albumin (monomeric)	66,000	0.4769
Ovalbumin	45,000	0.5872
Trypsinogen	24,000	0.7179
β-lactoglobulin	18,400	0.8051

b : Antigenic transglutaminase forms.

Transglutaminase form	Molecular weight	Kav
Rat fibrosarcoma		
Cytosolic	85,100 ± 800	0.3974±0.002
Particulate	104,700 ± 1100	0.3402±0.005
Inactive	120,000 ± 1700	0.3179±0.006
Hamster fibrosarcoma		
Cytosolic	81,200 ± 1000	0.4084±0.004
Particulate	102,000 ± 1300	0.3513±0.005
Inactive	117,000 ± 1600	0.3190±0.006

6.2.3 Immunoaffinity chromatography.

The affinity of the available polyclonal antibodies for the three antigenic forms of transglutaminase has already been discussed (section 4.4.2). However, the avidity with which the antibodies bind to the different forms is not known. In order to assess this avidity, and to ascertain whether or not immmunoaffinity chromatography could be used as a purification step, immunoaffinity columns were set up by coupling rabbit anti-rlct antibody to cyanogen bromide activated - Sepharose (section 2.8.3.1). Semi-pure preparations of the three forms, obtained by anion-exchange chromatography of lubrol extracts of P8 and Met B tissue, were loaded onto these columns and the protein then eluted with :-

1 PBS

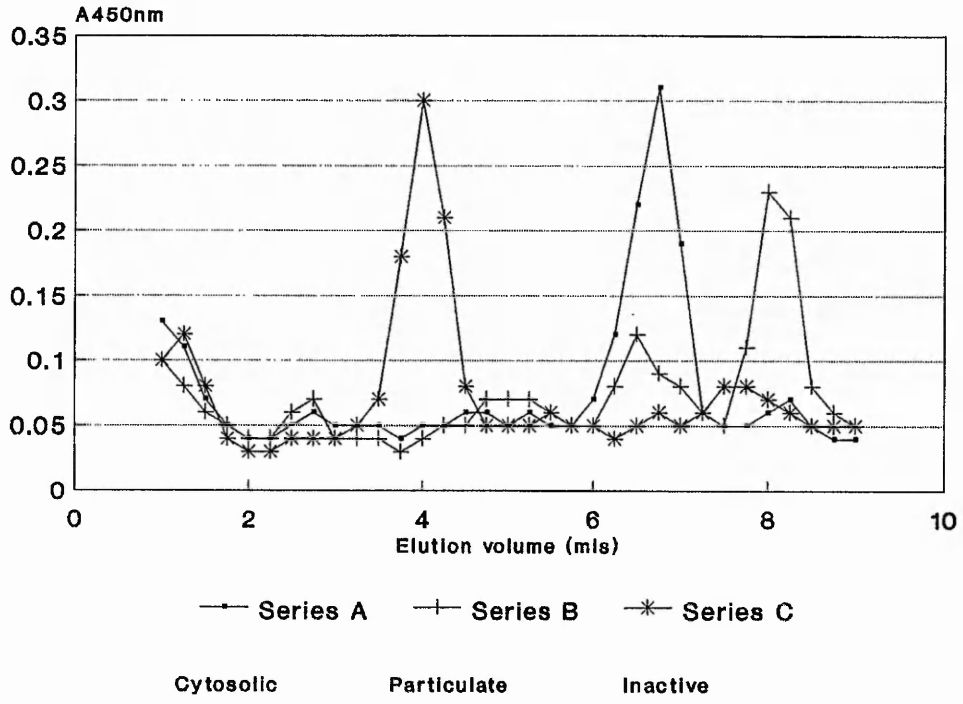
2 0.25M glycine-HCl pH2.5

3 0.25M glycine-HCl pH2.5 containing 10% (v/v) dioxane.

Comparison of the elution characteristics of the different antigenic forms (Fig.6.4) suggests that, although the affinity of the rabbit anti-rlct antibody for the inactive antigen is higher than for both the cytosolic and particulate forms, its avidity is lower. Similarly, unlike affinity, the avidity of the rabbit anti-rlct antibody for the particulate and the cytosolic forms of transglutaminase differs, with the particulate form showing an increased avidity over the cytosolic. They also indicate that immunoaffinity chromatography could be included as a step in a purification protocol for the different enzyme forms.

Figure 6.4 : Typical antigen profiles of the elution of the three forms of antigenic transglutaminase from an immunoaffinity column.

Semi-pure samples (>20% purity) (from anion-exchange chromatography) of each form of transglutaminase (~500ng) from the rat sarcoma P8, were applied and eluted from a cyanogen-bromide activated sepharose immunoaffinity column (7ml) as detailed in section 2.8.3.
Eluted fractions were assayed for transglutaminase antigen by a poly-l-lysine ELISA (section 2.6.3.2).


A450nm
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
2
4
6
8
10
Elution volume (mls)
Series A
Series B
Series C
Cytosolic
Particulate
Inactive

6.2.4 GTP-affinity chromatography.

Various reports have suggested that transglutaminase activity may be modulated by the binding of GTP to the enzyme (Achyuthan and Greenberg (1987); Bergamini et al (1987) and Bergamini (1988)). In order to ascertain if each of the three antigenic forms of tissue transglutaminase bind GTP, GTP-affinity chromatography (section 2.8.4) was utilised (Lee et al (1989)).

Known amounts of the three forms of immunoaffinity purified enzyme obtained from P8 and Met B tissue (section 6.2.3), were loaded onto a GTP-agarose column. The column was then washed with 100mM Tris-HCl pH7.5, 1mM EDTA (buffer A) until the eluent was free of protein, and any bound protein then eluted with buffer A containing 1M KCl, as detailed in section 2.8.4. Of the protein loaded onto the column, more than 80% bound, making it apparent that all three forms of transglutaminase, whether showing transglutaminase activity or not, were capable of binding to GTP (Table 6.2).

Table 6.2 : GTP-agarose chromatography of immunoaffinity purified transglutaminase forms from P8 rat sarcoma.

Enzyme form	Fraction	Transglutaminase ug/fraction
Particulate	Sample loaded	3.3
	Column run off	0.12
	Wash 1	N/D
	Wash 2	N/D
	Wash 3	N/D
	KCl wash 1	2.4
	KCl wash 2	0.41
	KCl wash 3	N/D
	KCl wash 4	N/D
	KCl wash 5	N/D
Inactive	Sample loaded	7.2
	Column run off	0.65
	Wash 1	0.15
	Wash 2	N/D
	Wash 3	N/D
	KCl wash 1	0.76
	KCl wash 2	4.81
	KCl wash 3	0.37
	KCl wash 4	0.18
	KCl wash 5	N/D
Cytosolic	Sample loaded	8.2
	Column run off	0.54
	Wash 1	0.2
	Wash 2	N/D
	Wash 3	N/D
	KCl wash 1	0.51
	KCl wash 2	3.51
	KCl wash 3	2.9
	KCl wash 4	0.3
	KCl wash 5	N/D

Samples (3 - 8 ug transglutaminase) were applied to a 3ml GTP-agarose column and eluted as described in section 2.8.4. Eluent fractions were assayed for antigen level by the quantitative ELISA described in section 2.6.3.1.

6.3 Purification of the particulate and inactive forms of transglutaminase from rat sarcomas.

Four liquid chromatography steps were carried out in order to purify the two forms of transglutaminase. These were :-

1 anion-exchange (section 2.8.1.2)

2 gel filtration (section 2.8.2.2)

3 immunoaffinity chromatography (section 2.8.3.2)

4 GTP-affinity chromatography (section 2.8.4)

Step 1 separates the transglutaminases from one another by utilising the difference in there ionic capacity; step 2 removes proteins of similar ionic capacity that differ in molecular size; step 3 utilises the binding of antigen to antibody, to remove contaminating proteins of similar molecular size and ionic strength; step 4 relies on the GTP binding properties of transglutaminase to remove any extraneous proteins that may cross-react with the antibody.

A typical protocol for purification is as follows :-

1 : Homogenisation and extraction.

Tissue (approximately 50g of P8 rat sarcoma) was homogenised to 20% (w/v) in ice cold homogenising buffer containing 1μg/ml pepstatin, 1μg/ml leupeptin, and 1% (w/v) Lubrol-PX. The homogenate was kept on ice on a horizontally rotating bed for 1h before being fractionated into supernatant and pellet by centrifugation at 71,000g for 45min at 4°C. A sample of the homogenate was kept on ice and, along with the supernatant and 71,000g pellet (resuspended in a volume of homogenising buffer equal to that of the supernatant) was assayed for

transglutaminase activity (Table 6.3).

2 : Anion-exchange chromatography.

Proteins in the 71,000g supernatant from (1) were separated on a 300ml Q - Sepharose column by elution with a 0 - 0.5 M NaCl gradient. Each eluent fraction (12ml) was assayed for transglutaminase activity and antigen as well as conductivity. Antigenic fractions (0.15 -0.22 M NaCl for particulate, 0.28 -0.32 M NaCl for inactive) were pooled and assayed for activity and antigen concentration (Table 6.3).

3 : Ammonium sulphate precipitation.

Pooled fractions from (2) were concentrated by the addition of ammonium sulphate to 60% saturation (to negate the detrimental effects of metal ion contamination, EDTA was also added to a final concentration of 20mM). The resultant precipitate was taken up in 1ml 10mM Tris-acetate pH6, 1mM EDTA, 0.16M KCl, and assayed for activity and antigen (Table 6.3).

4 : Gel filtration chromatography.

The concentrated pool from (3) was chromatographed on a 2.5 x 100 cm Ultragel ACA44 column. Eluent fractions (2.5ml) were assayed for activity and antigen, and the relevant antigenic peaks pooled and concentrated using an amicon with a 20,000D cut off.

5 : Immunoaffinity chromatography.

The concentrated pool from (4) (2ml) was applied to an immunoaffinity column (7ml) and the unbound protein washed off with PBS. Bound protein was then eluted with 10% (v/v) dioxane in 0.25M glycine pH2.5. Antigenic fractions were pooled, desalted on a Sephadex PD-10 column and concentrated by amicon as in (4).

6 : GTP-affinity chromatography.

The concentrate from (5) was applied to a GTP-agarose column (3ml) and unbound protein washed off with 100mM Tris-HCl pH7.5, 1mM EDTA. Bound protein was eluted by the addition of 1M KCl to the washing buffer. Pooled antigenic fractions were concentrated, and the buffer diluted to approximately 5mM Tris-HCl pH7.4, using an amicon with a 20,000 Mr cut off.

The purity of each form of transglutaminase at each stage in the protocol is given in Table 6.3.

Pure preparations of the particulate and inactive forms of transglutaminase obtained from the rat fibrosarcoma P8, harvested at 32 days growth, were used for all the remaining experiments in this chapter. The cytosolic transglutaminase used was purified (by the method of Connellan et al, 1971) from guinea pig liver, by Dr.D. Hand, Nottingham Polytechnic, unless otherwise stated.

Table 6.3 : Purification of particulate and inactive transglutaminase from P8 rat sarcoma.

Enzyme form	Stage	Total protein (mg)	S.Act. (U/mg protein)	ug Tg.	% purity
Particulate	Homogenate	6243	0.979		
	Pooled ion-exchange fractions	7.8	106.8	507	7
	Pooled gel filtration fractions	2.23	1065.6	452	20
	Immuno-affinity fraction	0.448	5298.3	414	92
	GTP-affinity fraction	.217	10951	271	100
Inactive	Homogenate	6243			
	Pooled ion-exchange fractions	34.9		586	6
	Pooled gel filtration fractions	2.91		442	23
	Immuno-affinity fraction	0.478		376	85
	GTP-affinity fraction	.347		325	94

Purification was carried out as detailed in section 6.3.

6.4 Proteolysis of the three forms of transglutaminase.

The presence of an inactive enzyme protein in the cytosol of metastatic tumours, possessing a molecular weight greater than that of the cytosolic enzyme, suggested the possibility of this protein being a precursor to the active cytosolic form. Such a hypothesis would agree with the data shown in Table 4.3 and Table 5.1, where decreases in the expression of the cytosolic form of the enzyme in highly metastatic hamster and rat sarcomas is accompanied by a corresponding increase in the level of the inactive enzyme protein.

Plishker et al (1978) reported that treatment of human epidermal transglutaminase with trypsin caused an increase in activity; Negi et al (1985) have reported the existence of an epidermal transglutaminase 22,000 daltons larger than the normal epidermal form, that could be cleaved into the normal form by a variety of proteases with a concomitant increase in activity; Chung et al (1988) have demonstrated the cleavage of the 95,000 dalton particulate tissue transglutaminase into a more active 50,000 dalton enzyme. These reports all suggest that transglutaminase enzymes are susceptible to activation by limited proteolysis.

Limited proteolysis using trypsin or thrombin was therefore undertaken on the particulate and inactive forms, from P8 fibrosarcoma, purified as in steps 1 - 6 of section 6.3, on purified guinea pig liver transglutaminase purified by the method of Connellan et al (1971) (courtesy of Dr. D. Hand, Nottingham Polytechnic) and on cytosolic transglutaminase (~85 % pure), from P8 fibrosarcoma, immunoaffinity purified following semi-purification by anion-exchange chromatography.

6.4.1 The effect of limited proteolysis on the activity of the three antigenic forms.

Limited proteolysis of the purified transglutaminases was performed at 37°C for 10min using 10ng trypsin or 0.5units thrombin per ug of transglutaminase. Trypsin inhibitor, 1ng/ng trypsin, was added to trypsinised samples before assessing transglutaminase activity. Samples of each form, without proteolytic enzymes, were also incubated at 37°C as controls.

Particulate transglutaminase specific activity, assayed by the incorporation of ^{14}C putrescine into N,N'-dimethylcasein, was increased by more than 100% over the initial value by the action of the proteolytic enzymes mentioned above. In contrast, the specific activities of both the guinea pig cytosolic form, and the P8 cytosolic form, after proteolysis were only ~10% of their respective initial values, which were comparable. Partial proteolysis of the inactive form lead to its activation with an estimated specific activity similar to that of the normal cytosolic and particulate forms (Table 6.4). Incubation alone caused decreases in specific activity to both the cytosolic and particulate forms.

6.4.2 The active enzyme formed by limited proteolysis of the inactive antigen is a tissue transglutaminase.

A simple definition of a transglutaminase is a calcium dependent enzyme with an active site thiol, catalysing an acyl transfer

Table 6.4 : Comparison of the specific activity of the three transglutaminase forms after partial proteolysis.

Purified enzymes (8ug in 100ul) were assayed by ^{14}C-putrescine incorporation into N,N'-dimethylcasein to give initial activities.

Aliquotes (25ul) were then removed and incubated at 37^{o}C for 10min :-

a) without the addition of a proteolytic enzyme;

b) with the addition of 20ng of trypsin;

c) with the addition of 1unit of thrombin.

After incubation, samples were assayed for transglutaminase activity by the ^{14}C-putrescine assay (section 2.6.1.1).

Trypsin inhibitor (1ng/ng trypsin) was added to trypsinised samples prior to assaying transglutaminase activity.

1 - refers to cytosolic transglutaminase purified from guinea pig liver.

2 - refers to cytosolic transglutaminase purified from the P8 rat sarcoma.

Particulate and inactive forms were purified as detailed in section 6.3; guinea pig liver cytosolic transglutaminase was purified by the method of Connellan et al, 1971, courtesy of Dr. D. Hand; P8 rat sarcoma cytosolic transglutaminase was semi-purified (>95%) by anion-exchange (section 2.8.1.1) followed by immunoaffinity chromatography (section 2.8.3.2).

Treatment	Enzyme form	Specific Activity (U/ug transglutaminase)
Initial activity	Particulate	9.5 ± 3.2
	Cytosolic [1]	11.2 ± 2.7
	Cytosolic [2]	9.8 ± 2.1
	Inactive	
Incubation at 37°C for 10min	Particulate	4.6 ± 1.8
	Cytosolic [1]	2.8 ± 0.6
	Cytosolic [2]	2.2 ± 0.6
	Inactive	
Incubation at 37°C for 10min with trypsin	Particulate	24.0 ± 2.0
	Cytosolic [1]	1.4 ± 0.6
	Cytosolic [2]	0.8 ± 0.2
	Inactive	9.1 ± 0.8
Incubation at 37°C for 10min with thrombin	Particulate	19.7 ± 1.5
	Cytosolic [1]	1.1 ± 0.4
	Cytosolic [2]	0.9 ± 0.3
	Inactive	7.8 ± 1.1

reaction between primary amine groups and peptide-bound glutamine residues. Tissue transglutaminase, unlike epidermal transglutaminase, is heat labile. Thus if the active proteolytic fragment of the inactive antigen is a tissue transglutaminase, its ability to incorporate polyamines into proteins - ie. putrescine into N,N'-dimethylcasein - must be susceptible to chelating agents, such as EDTA and EGTA, to the absence of DTT and to temperature, and must be sensitive to calcium. To obtain proof that this active fragment was indeed a tissue transglutaminase a number of experiments were undertaken as listed below. The transglutaminases used were pure preparations of the three antigenic forms. Enzyme fragments were obtained by the limited proteolysis, with trypsin, of these pure preparations.

6.4.2.1 The effect of the presence of chelating agents on activity.

Activity was assessed by the incorporation of ^{14}C-putrescine into N,N'-dimethylcasein in the presence of 10mM EDTA or 10mM EGTA. The values obtained were compared to those for the same enzymes in the presence of 2.5mM calcium chloride. All forms were found to require the presence of calcium to show activity. Specific activity in the presence of either EGTA or EDTA, was found to be less than 10% of that with calcium present (Table 6.5).

6.4.2.2 The requirement of the enzymes for the reducing agent DTT.

When transglutaminase activity was measured in the presence and absence of DTT it was found that the presence of this reducing

Table 6.5 : Specific activity of transglutaminase forms in the presence or absence of chelating agents and dithiothreitol.

Purified enzymes (25 ul aliquots of a solution of 6ug in 75ulTE) and partially proteolysed purified enzymes (25ul aliquots of a solution of 6ug in 75ulTE, partially proteolysed by incubation for 10min at 37°C in the presence of 6ng of trypsin) were assayed by ^{14}C-putrescine incorporation into N,N'-dimethylcasein (section 2.6.1.1) in the presence of :-

a) Ca^{2+} (3mM) + DTT (7.7mM);

b) EGTA (10mM) + DTT (7.7mM);

c) EDTA (10mM) + DTT (7.7mM);

d) Ca^{2+} (3mM) - DTT;

Trypsin inhibitor (1ng/ng trypsin) was added to trypsinised samples prior to assaying transglutaminase activity.

1 - refers to cytosolic transglutaminase purified from guinea pig liver.

2 - refers to cytosolic transglutaminase purified from the P8 rat sarcoma.

Particulate and inactive forms were purified as detailed in section 6.3; guinea pig liver cytosolic transglutaminase was purified by the method of Connellan et al, 1971, courtesy of Dr. D. Hand; P8 rat sarcoma cytosolic transglutaminase was semi-purified (>95%) by anion-exchange (section 2.8.1.1) followed by immunoaffinity chromatography (section 2.8.3.2).

Enzyme form	Control ($+Ca^{2+}$)	EGTA	EDTA	-DTT
Particulate	11.0 ± 1.3	0.9 ± 0.2	1.4 ± 0.7	1.3 ± 0.8
Cytosolic [1]	9.3 ± 0.9	0.7 ± 0.4	1.0 ± 0.6	1.2 ± 0.5
Cytosolic [2]	7.9 ± 1.1	0.6 ± 0.3	1.3 ± 0.7	1.0 ± 0.4
Proteolysed particulate	23.2 ± 3.2	1.7 ± 1.1	2.7 ± 0.7	2.8 ± 1.1
Proteolysed cytosolic	1.4 ± 0.4	0.6 ± 0.2	0.4 ± 0.2	0.4 ± 0.1
Proteolysed inactive	7.8 ± 1.0	0.9 ± 0.3	1.2 ± 0.5	1.3 ± 0.4

agent was necessary to maintain the activity of all the forms capable of incorporating putrescine into N,N´-dimethylcasein. Less than 15% of the activity was found in the absence of DTT (Table 6.5).

6.4.2.3 Enzyme stability at 37°C.

Samples were incubated at 37°C and aliquots removed at 0, 5, 10, 20 and 30 min. These aliquots were assayed for transglutaminase activity and the levels found expressed as a percentage of the initial activity. All enzyme forms were found to be heat labile; the proteolysed particulate form showed the most stability, losing only 30% activity, with the particulate, cytosolic and proteolysed inactive losing 60 - 70% (Fig.6.5).

6.4.2.4 Estimation of sensitivity to Ca^{2+} activation.

Each of the active forms of tissue transglutaminase (cytosolic, particulate, partially trypsinised particulate and partially trypsinised inactive) were assayed for their sensitivity to Ca^{2+}. The method used was essentially that of Hand et al (1985) which measures the incorporation of ^{14}C-putrescine into dephosphorylated N,N´ dimethylcasein. Enzyme activity was measured at calcium concentrations ranging from 5.47 - 100 uM. The apparent Km of each enzyme was then calculated from a Lineweaver-Burk plot (Lineweaver and Burk 1934) (Fig.6.6).

The sensitivity of each enzyme form to calcium is shown in Fig.6.7, and the calculated Km´s shown in Table 6.6. From these

Figure 6.5 : Stability of enzyme activity at 37°C.

Enzyme activity at 37°C was monitored over a 30min time period. Duplicate samples (~10ug in 150ul TE) were incubated at 37°C, and duplicate 12ul aliquotes removed at 0, 5, 10, 20 and 30 min for assessment of activity. Activity was assayed by the calcium dependant incorporation of ^{3}H-putrescine into N,N'-dimethylcasein (section 2.6.1.2).

Cytosolic transglutaminase was obtained from guinea pig liver; particulate and inactive transglutaminase were obtained from the rat sarcoma P8. Activated forms of particulate and inactive transglutaminase were obtained by partial proteolysis with trypsin at 37°C for 10min using 10ng trypsin per ug of transglutaminase. Trypsin inhibitor, 1ng/ng trypsin, was added to trypsinised samples before assessing transglutaminase activity.

Activity is expressed as a percentage of the activity present at time 0.

Data represents the mean of 3 experiments + SE.

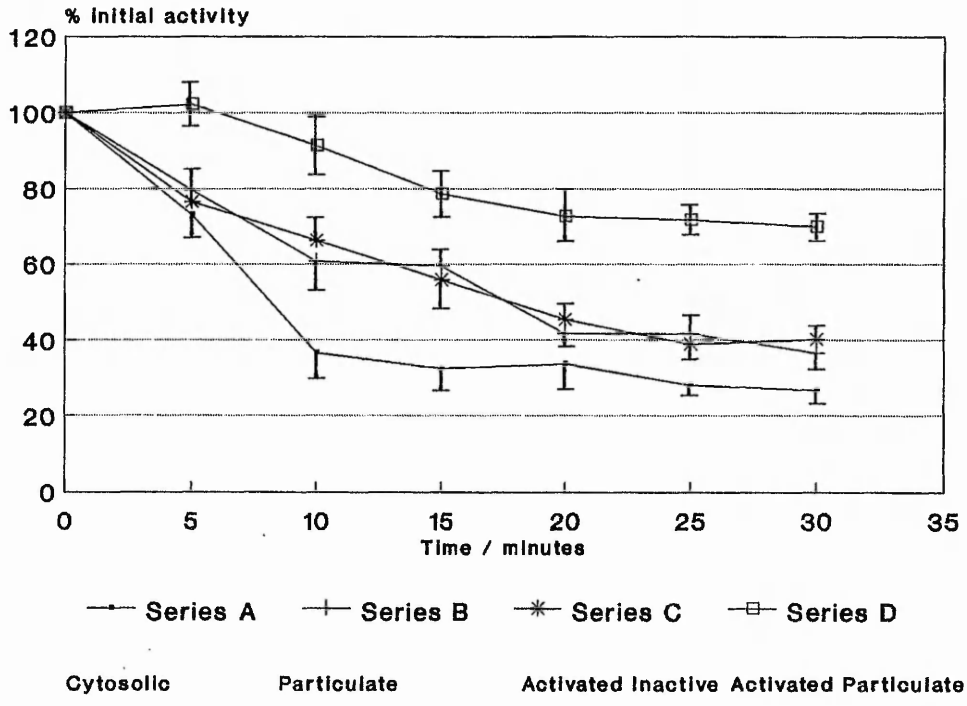

% initial activity
120
100
80
60
40
20
0
0
5
10
15
20
25
30
35
Time / minutes
Series A
Series B
Series C
Series D
Cytosolic
Particulate
Activated
Inactive
Activated
Particulate

Figure 6.6 : Representative Lineweaver-Burk plots for the sensitivity of the active forms of transglutaminase to Calcium.

The apparent Km for calcium sensitivity was estimated by varying the concentration of calcium in a modification of the transglutaminase assay that replaced N'N'-dimethylcasein with a dephosphorylated form (courtesy of Dr. D. Hand, Nottingham Polytechnic).

Transglutaminase was measured as detailed in section 2.6.1.1, except that the following conditions were utilised (Hand et al (1985)

reaction vials contained :-

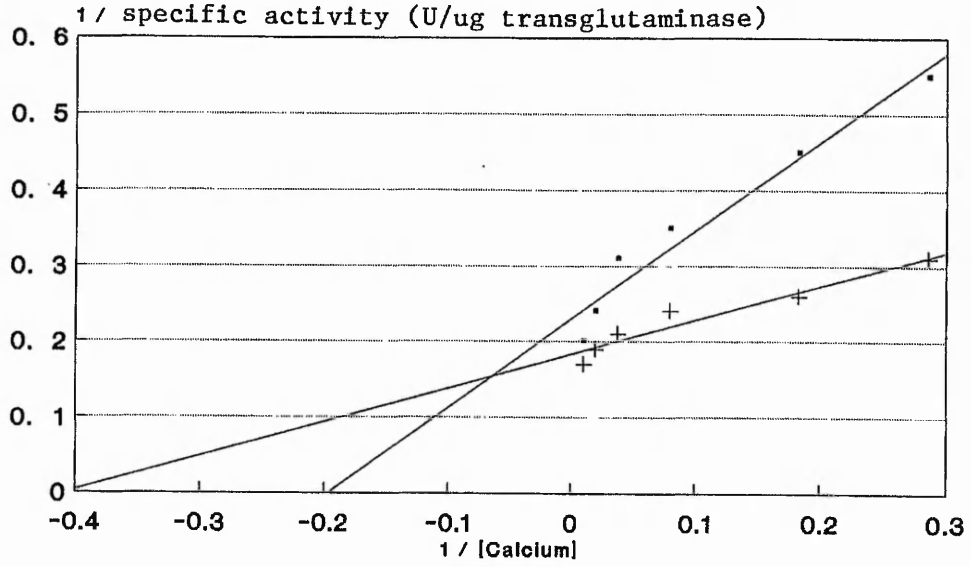
[1,4-^{14}C]-putrescine (3.97mCi/mmol)	1.20mM
DTT	4.05mM
dephosphorylated N,N'-dimethylcasein	5mg/ml
magnesium chloride	2.00mM
EGTA (in sample)	0.40mM
Tris-HCl pH7.4 at 37^{o}C	50.0mM
calcium chloride (0.4 - 2.5 mM)	5.47 - 2100.01 uM free Ca^{2+}

Sample (20ul (~2ug transglutaminase) containing 1mM EGTA) was added to give a final volume of 50ul.

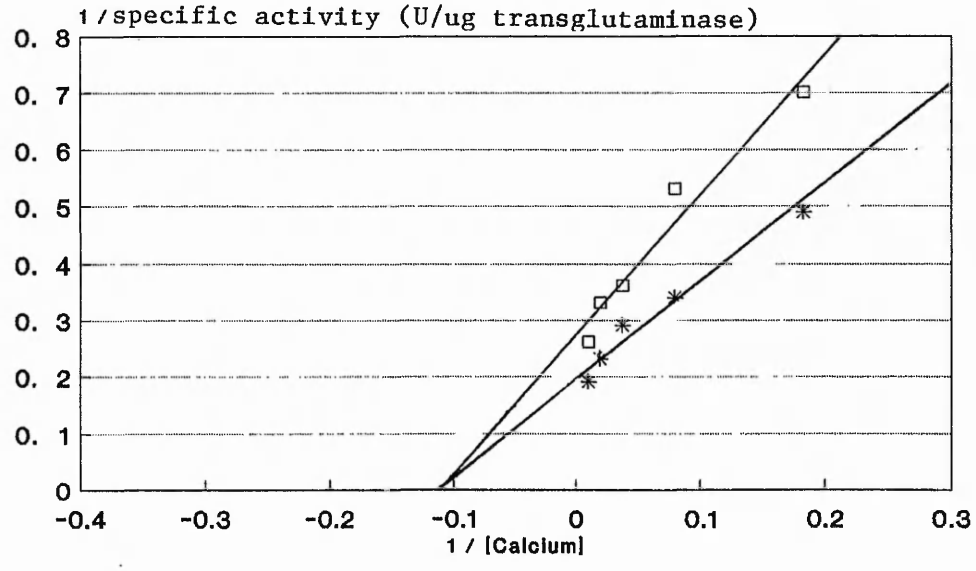
Free calcium concentrations were calculated, using an association constant for Ca/EGTA of $10^{7.12}$ at pH7.4 and 37^{o}C, by the method of Schatzmann (1973) (Hand et al (1985)).

Particulate and inactive transglutaminase forms (>95% pure) were obtained from P8 rat sarcoma, cytosolic transglutaminase was from guinea pig liver.

Activation of the particulate and inactive forms was by partial trypsinisation at 37^{o}C as detailed in the legend to Fig. 6.5.



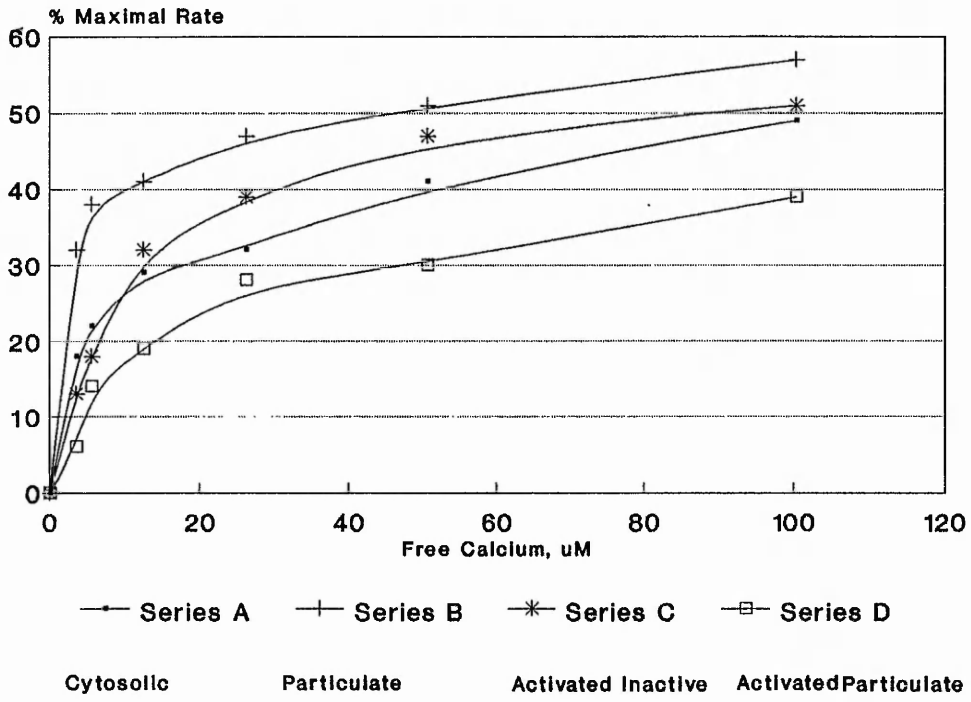
Cytosolic Particulate



Activated Inactive Activated Particulate

Figure 6.7 : The sensitivity of active tissue transglutaminases to calcium.

Transglutaminase was measured as detailed in the legend to Fig. 6.6. Free calcium concentrations (5.47 - 100 uM) were calculated as described in the legend to Fig. 6.6.


% Maximal Rate
60
50
40
30
20
10
0
0
20
40
60
80
100
120
Free Calcium, uM
Series A
Series B
Series C
Series D
Cytosolic
Particulate
Activated Inactive
ActivatedParticulate

results, it is apparent that the two active proteolysed forms are less sensitive to calcium than the normal active particulate and cytosolic forms, although similar to each other. The lower Km apparent for the uncleaved particulate form would suggest that it is more sensitive to calcium than the cytosolic form.

Table 6.6 : Sensitivity of transglutaminases to calcium activation.

Transglutaminase form	Km_{app} (uM)
Cytosolic	4.4 ± 0.82
Particulate	2.6 ± 0.53
Proteolysed Particulate	8.5 ± 1.16
Proteolysed Inactive	5.8 ± 1.01

Transglutaminase activity was measured at various concentrations of free calcium (5.47 - 100 uM) as detailed above. Km_{app} was estimated from double reciprocal plots (Lineweaver-Burk) after extrapolation.

Data is given as the mean value of 3 determinations ± SE.

6.4.3 Comparison of the active forms.

Further characterisation of the proteolysed inactive transglutaminase was undertaken by three different methods. The first involved separation of the proteolysed mixture by anion-exchange chromatography. Secondly, the molecular weight of the proteolysed fragments was estimated by gel filtration. Thirdly, western blots of the proteolysed fragments, run on polyacrylamide gels, were probed with anti-cytosolic transglutaminase antibodies.

The active proteolysed forms used in this section came from the treatment of immunoaffinity purified inactive transglutaminase with 10ng trypsin for 10min at 37°C (section 6.4.1).

6.4.3.1 Anion-exchange chromatography of the proteolysed forms.

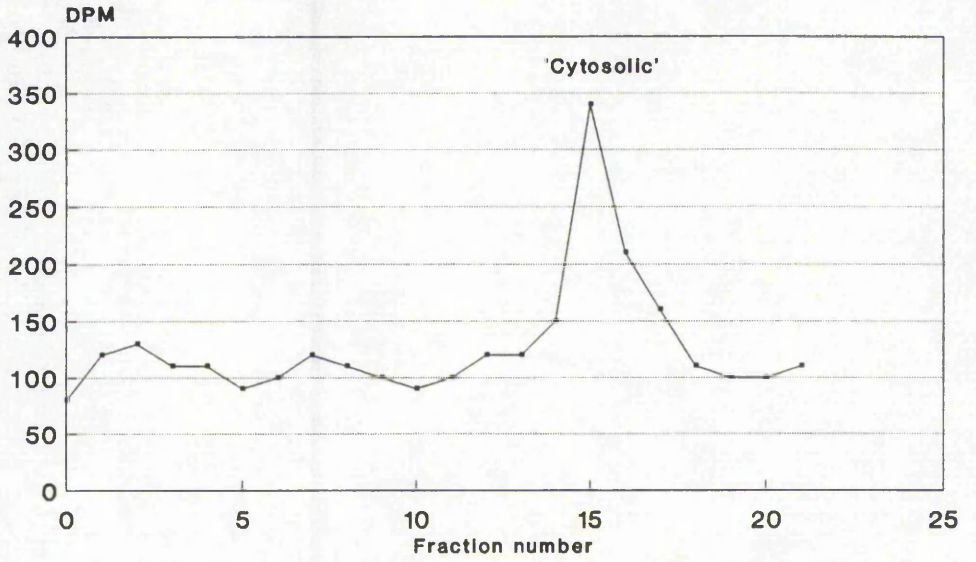
Crude proteolysed inactive antigenic transglutaminase was subjected to anion-exchange chromatography and the eluent fractions assayed for transglutaminase activity and antigen. Partial proteolysis of the inactive antigen gave rise to the presence of two antigenic peaks, one eluting at 0.25 - 0.3 M NaCl corresponding to the inactive enzyme protein, and one eluting at 0.35 - 0.45 M NaCl corresponding to the cytosolic enzyme; and one active peak eluting at 0.35 - 0.45 M NaCl, corresponding to the cytosolic antigen peak (Fig.6.8).

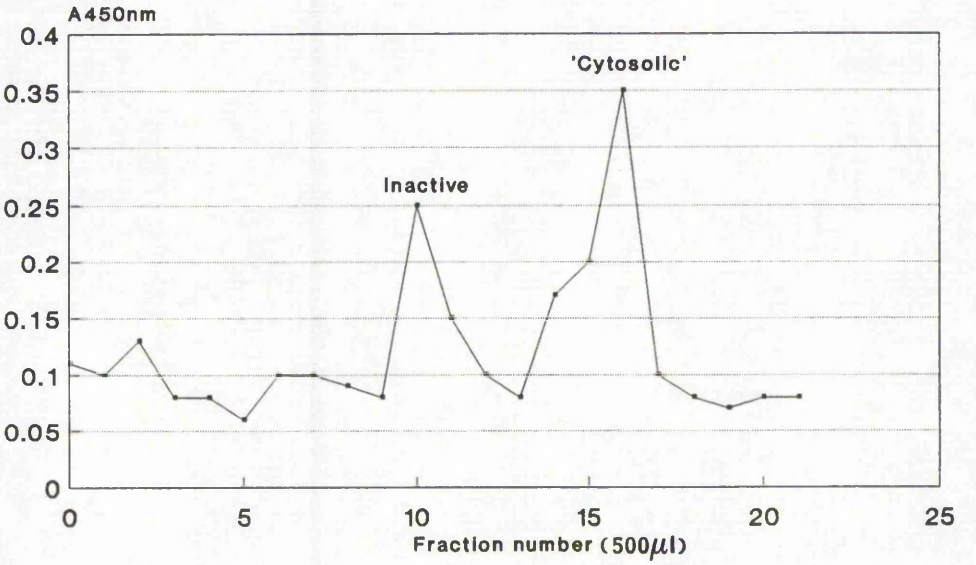
Figure 6.8 : Anion-exchange elution profile for the activated form of the inactive transglutaminase protein.

Inactive transglutaminase (~500ng) from the P8 rat sarcoma was activated by partial trypsinisation at 37°C for 10min as detailed in the legend to Fig. 6.5. The activated form (~500ng prootein) was then chromatographed on a Pharmacia Mono-Q (1ml) anion-exchange column (section 2.8.1.1).

a) Activity profile : eluent fractions were assayed for transglutaminase activity by the calcium dependent incorporation of ^{3}H-putrescine into N'N'-dimethylcasein (section 2.6.1.2).

b) Antigen profile : eluent fractions were assayed for antigenic transglutaminase by a poly-l-lysine ELISA (section 2.6.3.2).


DPM
400
350
300
250
200
150
100
50
0
'Cytosolic'
0
5
10
15
20
25
Fraction number


A450nm
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
'Cytosolic'
Inactive
0
5
10
15
20
25
Fraction number (500μl)

6.4.3.2 Molecular weight analysis.

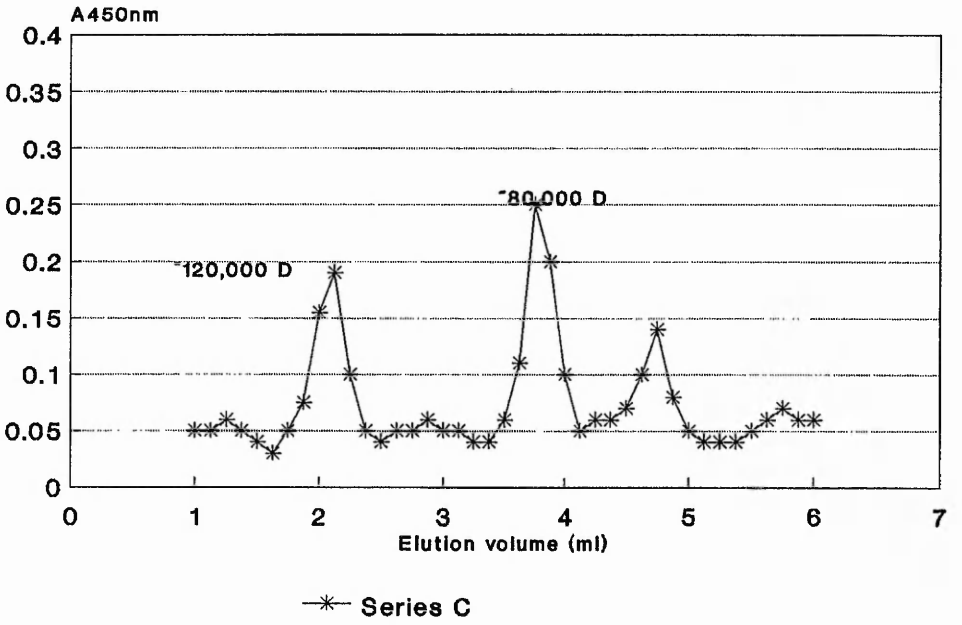
Gel filtration chromatography, on a pre-calibrated TSK 3000 SW molecular seive column (section 2.8.2.1 and section 6.2.2), of the crude proteolysis preparation enabled the estimation of the molecular weight of the proteolysed fragments. Limited proteolysis of the inactive transglutaminase antigen produced three detectable antigenic peaks. One with a Mr of 120,000 daltons corresponded to the inactive antigen, a major peak which appeared at 80,000 daltons, corresponding to the elution of the cytosolic enzyme (section 6.2.2), and a further minor peak which eluted with an Mr of approximately 65,000 daltons (Fig.6.9).

6.4.3.3 Western blots of cytosolic, particulate and activated inactive antigen.

Confirmation that activation of the inactive antigen, by its partial proteolysis, led to the appearance of an enzyme protein mimicking the cytosolic form, was obtained by immunoprobing western blots of non-denaturing polyacrylamide gels of proteolysed inactive antigen, with goat anti-gplt antibody and anti-Factor XIII antibody. Comparison of the lane corresponding to the partially trypsinised inactive antigen with that for the cytosolic enzyme indicated the presence of identical bands, when probed with the anti-cytosolic antibody, but no reaction was observed with the anti-Factor XIII antibody (Fig.6.10).

Figure 6.9 : Typical antigen profile of the elution of the activated form of the inactive transglutaminase protein from a TSK 3000 SW gel filtration column.

Inactive transglutaminase (~500ng) from the P8 rat sarcoma was activated by partial trypsinisation at 37°C for 10min as detailed in the legend to Fig. 6.5. The activated form (~500ng) was then chromatographed on an Ultragel TSK 3000 SW gel filtration column (26.5ml) (Pharmacia-LKB) as detailed in section 2.8.2.1. Eluent fractions were assayed for transglutaminase antigen by the poly-l-lysine ELISA (section 2.6.3.2).


A450nm
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
120,000 D
80,000 D
0
1
2
3
4
5
6
7
Elution volume (ml)
Series C

Inactive

Figure 6.10 : Western blot of cytosolic, particulate, inactive and activated inactive transglutaminase.

Samples (~10ug per lane) of the transglutaminase forms, semi-purified (~25%) by anion-exchange, and the partially proteolysed activated inactive form (activated as described in the legend to Figure 6.5), were electrophoresed under non-denaturing conditions on a 7.5% (w/v) polyacrylamide resolving gel (section 2.5.2). Separated proteins were then electoblotted onto nitrocellulose from the polyacrylamide gel (section 2.5.3.2). Western blots were blocked with 2% (w/v) Marvel in TBS as in section 2.5.4 and then probed, for 2h at room temperature, with a 1/1000 dilution of goat anti-guinea pig liver transglutaminase antibody in TBS. Blots were developed using biotin conjugated anti-species antibodies (diluted 1/500 in TBS), followed by HRP labelled avidin (diluted 1/5000 in TBS) and chloronapthol reagent as in section 2.5.4.

Similar blots were probed with rabbit anti-Factor XIII antibody (diluted 1/1000 in TBS), but no reaction was seen.

p : particulate

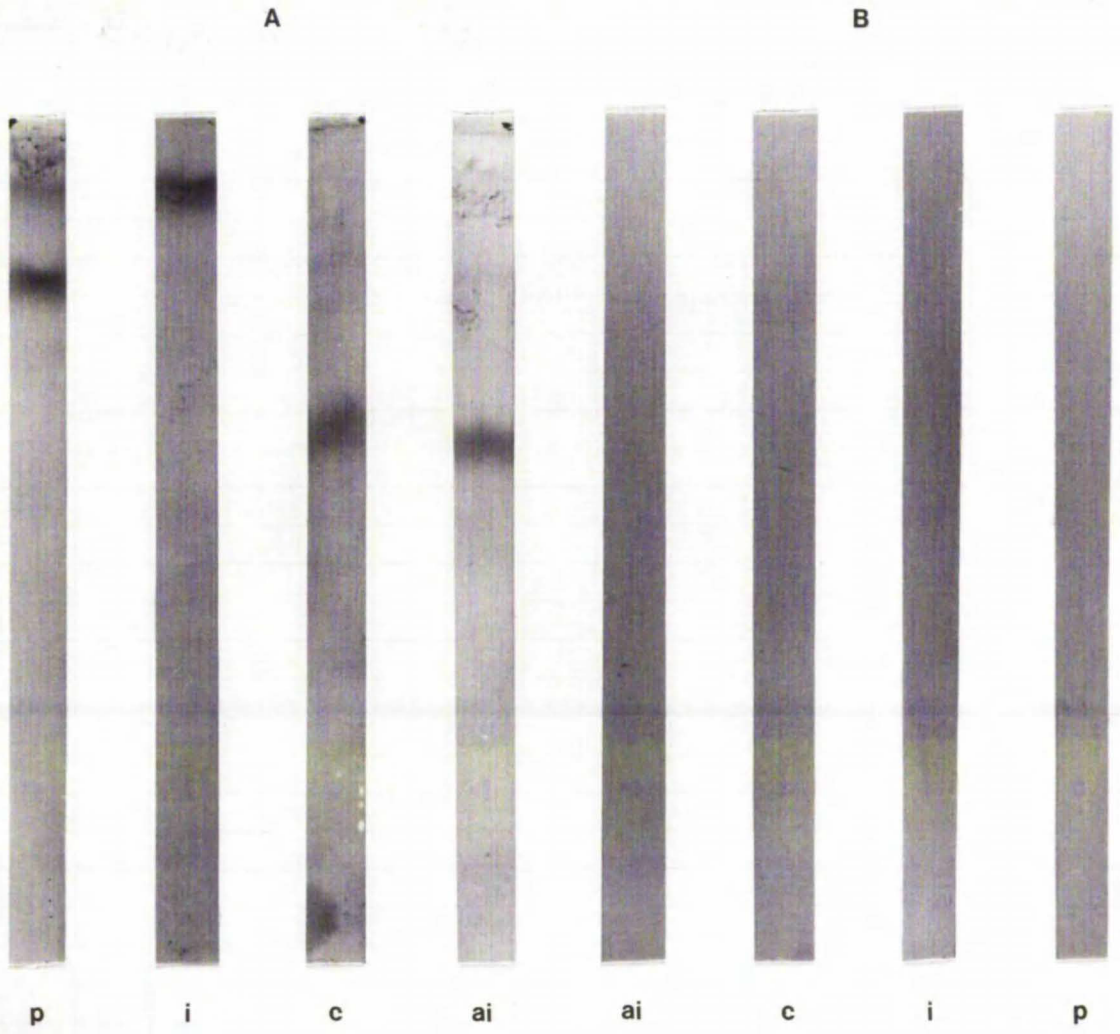
i : inactive antigen

c : cytosolic

ai : activated inactive antigen

A : probed with goat anti-gplt antibodies

B : probed with rabbit anti-Factor XIII antibodies.


A
B
p
i
c
ai
ai
c
i
p

6.4.3.4 Glycosylation of the three forms.

Western blots of the three forms and the activated inactive antigen, separated by non-denaturing polyacrylamide gel electrophoresis were also probed with the HRP labelled lectins Concanavalin A, Triticum vulgaris and Bandeiraea simplicifolia, in Tris buffered saline (TBS) (100mM Tris-HCl,pH7.4, 150mM NaCl) containing 10mM $CaCl_2$, 10mM $MgCl_2$ and 10mM $MnCl_2$. No reaction was observed between any of these lectins and the different transglutaminase forms, indicating that mannose, glucose and galactose, respectively, were not components of any of the enzyme forms (Fig.6.11).

Figure 6.11 : Lectin probed western blots of the three forms of tissue transglutaminase.

Samples (~10ug per lane) of the transglutaminase forms, semi-purified (>25%) by anion-exchange, and the partially proteolysed activated inactive form (activated as described in the legend to Figure 6.5), were electrophoresed under non-denaturing conditions on a 7.5% (w/v) polyacrylamide resolving gel (section 2.5.2). Separated proteins were then electoblotted onto nitrocellulose from the polyacrylamide gel (section 2.5.3.2). Western blots were blocked with 2% (w/v) Marvel in TBS as in section 2.5.4 and then probed, for 2h at room temperature, with the HRP labelled lectin Concanavalin A diluted 1/100 with TBS containing 10mM $CaCl_2$, 10mM $MgCl_2$, 10mM $MnCl_2$ (similar results were obtained when using the HRP labelled lectins Triticum vulgaris and Bandeiraea simplicifolia at the manufacturers recommended dilution). Blots were then developed using chloronapthol reagent as in section 2.5.4. The glycoprotein was included on the western blot as a control.

c : cytosolic

p : particulate

i : inactive antigen

ai : activated inactive antigen

g : glycoprotein (?)


c
p
g
i
ai

6.4.4 Comparison of tryptic digests of the three forms.

Since polyclonal antibodies raised against the cytosolic form of tissue transglutaminase also cross-react with the particulate and inactive forms, there must be some epitopes that are common to all three forms. Digestion of the three proteins into constituent peptides would give peptide maps for each form that could be probed with antibodies and compared for similar regions.

Purified preparations of the three forms were subjected to digestion with trypsin (10ng per ug transglutaminase) at 37^{o}C for 72h. Digested material was then electrophoresed on 10% (w/v) SDS polyacrylamide gels. Gels were then western blotted onto nitrocellulose and immunoprobed with goat anti-gplt or rabbit anti-rlct antibodies.

Western blots immunoprobed with rabbit anti-rlct (Fig.6.12) indicated a number of peptides containing common, or similar, epitopes to be present in the cytosolic and inactive maps. The extra bands present in the inactive map presumably cause the increase in affinity of the antibodies to the antigen. The immunoprobed peptide map of the particulate showed less than 30% homology to that of the cytosolic, yet both enzymes show similar affinity for the antibodies.

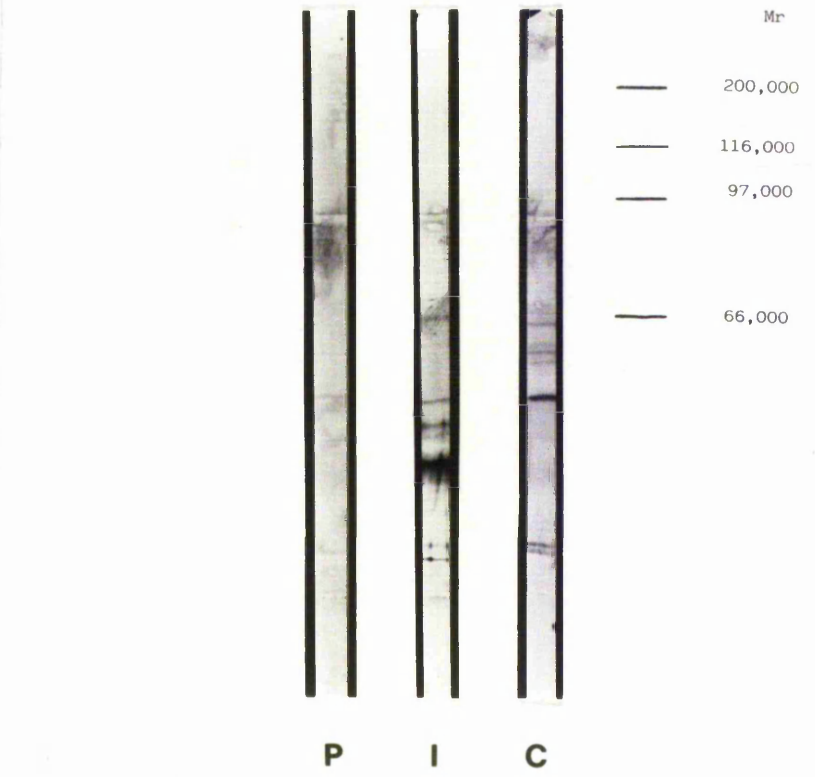
Figure 6.12 : Western blots of tryptic digests of the three forms of tissue transglutaminase.

Purified samples (~20ug) of particulate and inactive transglutaminase from the rat sarcoma P8 (section 6.3), and immunoaffinity purified (~95% pure) cytosolic transglutaminase from the rat sarcoma P8 (section 6.2.3) were digested by trypsin (20ng) at 37°C for 72h. After the addition of trypsin inhibitor (20ng), the digested material was electrophoresed on a 10% (w/v) polyacrylamide gel containing SDS (0.8% (w/v)). Separated proteins were then electoblotted onto nitrocellulose from the polyacrylamide gel (section 2.5.3.1). Western blots were blocked with 2% (w/v) Marvel in TBS as in section 2.5.4 and then probed, for 2h at room temperature, with a 1/1000 dilution of goat anti-guinea pig liver transglutaminase antibody in TBS. Blots were developed using biotin conjugated anti-species antibodies (diluted 1/500 in TBS), followed by HRP labelled avidin (diluted 1/5000 in TBS) and chloronapthol reagent as in section 2.5.4.

p : particulate

i : inactive

c : cytosolic


Mr
200,000
116,000
97,000
66,000
P
I
C

6.5 The association of lipid and proteinaceous material with the three forms.

Antigen profiles of anion-exchange chromatography eluent fractions indicated that both affinity purified goat anti-cytosolic transglutaminase and rabbit anti-cytosolic transglutaminase cross-reacted with the particulate form and the inactive antigen (section 4.4). However, if a monoclonal antibody raised against guinea pig liver (cytosolic) transglutaminase (courtesy of Dr.P.J.Birckbichler, Biomedical Division, The Samuel Roberts Noble Foundation, Inc., Ardmore, Oklahoma, USA.) was used as either the capture antibody or the visualising antibody in the sandwich ELISA, or as the visualising antibody in the poly-l-lysine ELISA, only the cytosolic and inactive peaks were observed (Fig.6.13). This indicated that the monoclonal anti-cytosolic antibody did not cross-react with the particulate enzyme in its native form, and gave further evidence to support the proposed relationship between the cytosolic and inactive forms.

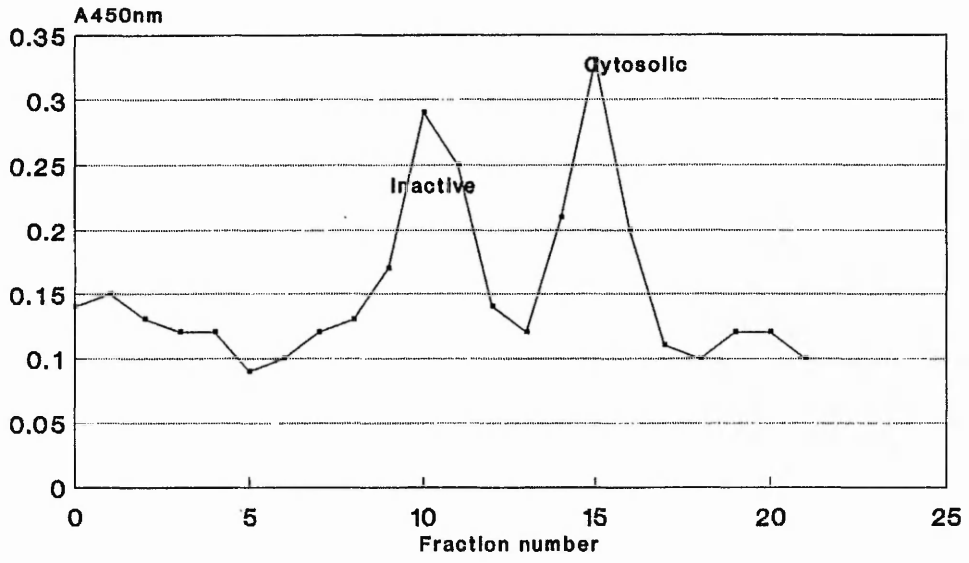
Western blots of non-denaturing polyacrylamide gels, immunoprobed with monoclonal anti-cytosolic transglutaminase, again only demonstrated cross-reactivity with the cytosolic and inactive forms (Fig.6.14). However, immunoprobing western blots obtained from denaturing SDS-polyacrylamide gels with the monoclonal antibody, gave rise to the demonstration of the cytosolic, inactive and particulate forms. The plasma transglutaminase Factor XIII, included as a control, did not cross-react with the monoclonal antibody (Fig.6.15). Furthermore, the relative mobilities and calculated molecular weights of the cytosolic and particulate forms (87,000 daltons in each case) were found to be comparable. The inactive form was calculated to be

Figure 6.13 : Anion-exchange chromatography antigen profiles using monoclonal anti-guinea pig liver transglutaminase antibody.

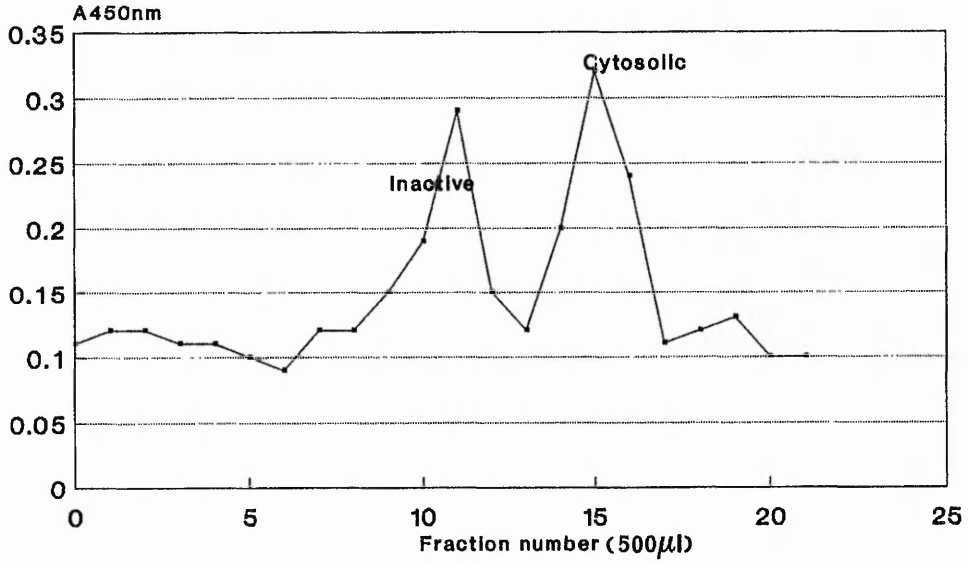
Non-necrotic P8 rat sarcoma tissue (500mg) was homogenised to give a final 20% (w/v) homogenate in homogenising buffer containing 1% (w/v) Lubrol-PX. Protein (approximately 10mg, equivalent to approximately 400ng total transglutaminase antigen) was loaded onto a Mono-Q column and eluted as in the legend to Fig.6.1.

a) Fractions, 500ul, were collected and assayed for antigenic transglutaminase by a modification of the sandwich ELISA (section 2.6.3.2) in which a 1/1000 dilution of monoclonal anti-guinea pig liver transglutaminase was used as the visualising antibody to give antigen elution profiles.

b) Fractions, 500ul, were collected and assayed for antigenic transglutaminase by a modification of the poly-l-lysine ELISA (section 2.6.3.2) in which a 1/1000 dilution of monoclonal anti-guinea pig liver transglutaminase was used as the visualising antibody to give antigen elution profiles.



Sandwich ELISA, monoclonal as capture



Poly-l-lysine, using monoclonal antibody

Figure 6.14 : Western blot of non-denatured tissue transglutaminase forms probed with monoclonal anti-guinea pig liver cytosolic transglutaminase antibodies.

Samples (~10ug per lane) of the transglutaminase forms, semi-purified (~25%) by anion-exchange, and the partially proteolysed activated inactive form (activated as described in the legend to Figure 6.5), were electrophoresed under non-denaturing conditions on a 7.5% (w/v) polyacrylamide resolving gel (section 2.5.2). Separated proteins were then electoblotted onto nitrocellulose from the polyacrylamide gel (section 2.5.3.2). Western blots were blocked with 2% (w/v) Marvel in TBS as in section 2.5.4 and then probed, for 2h at room temperature, with a 1/100 dilution of mouse monoclonal anti-guinea pig liver transglutaminase antibody in TBS. Blots were developed using biotin conjugated anti-species antibodies (diluted 1/500 in TBS), followed by HRP labelled avidin (diluted 1/5000 in TBS) and chloronapthol reagent as in section 2.5.4.

p : particulate

i : inactive antigen

c : cytosolic

ai : activated inactive antigen

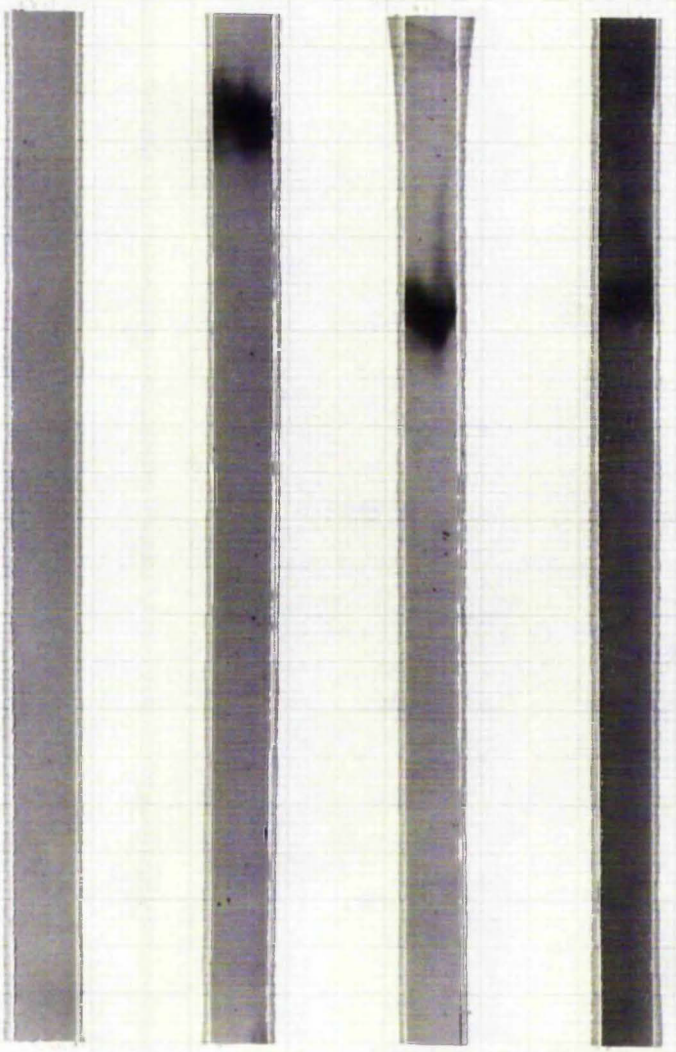
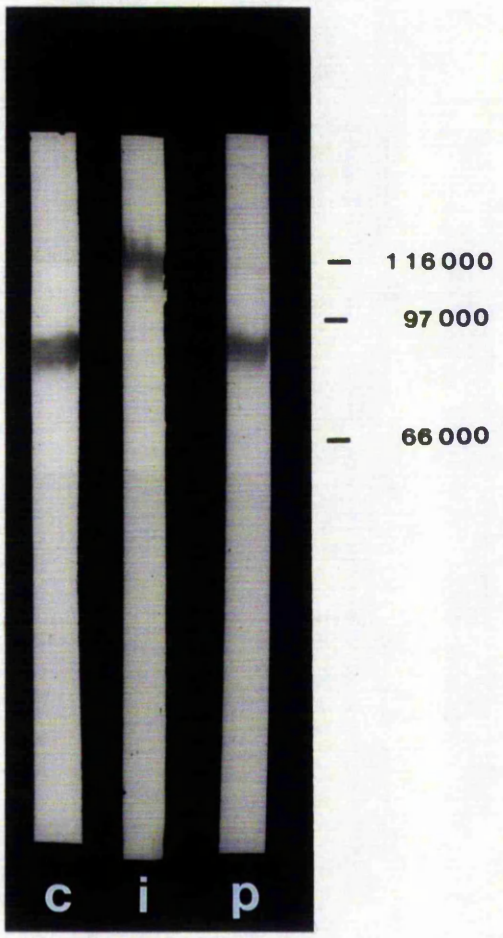

p
i
c
ai

Figure 6.15 : Western blot of cytosolic and particulate tissue transglutaminase.

Samples (~10ug per lane) of the particulate and cytosolic tissue transglutaminase forms, semi-purified (~25%) by anion-exchange, were electrophoresed under denaturing conditions on a 10% (w/v) polyacrylamide resolving gel containing 0.8% (w/v) SDS (section 2.5.1). Separated proteins were then electoblotted onto nitrocellulose from the polyacrylamide gel (section 2.5.3.1). Western blots were blocked with 2% (w/v) Marvel in TBS as in section 2.5.4 and then probed, for 2h at room temperature, with a 1/100 dilution of mouse monoclonal anti-guinea pig liver transglutaminase antibody in TBS. Blots were developed using biotin conjugated anti-mouse antibodies (diluted 1/500 in TBS), followed by HRP labelled avidin (diluted 1/5000 in TBS) and chloronapthol reagent as in section 2.5.4.

c : cytosolic

p : particulate


116000
97000
66000
c
i
p

approximatly 115,000 daltons which is comparable to that previously estimated by gel filtration (Table 6.1b; Fig. 6.3).

These results hinted at the possibility that the epitope for the monoclonal anti-cytosolic antibody, although present on the particulate form, was not recognisable in the proteins native conformation. Either the configuration of the protein was such as to render the epitope unrecognisable, or material was bound to the protein chain in such a way that the epitope was masked. To answer the question, what 'masks' the epitope, western blots of non-denaturing gels were treated with various enzymes prior to being immunoprobed with the monoclonal antibody.

6.5.1 The effect of lipase, collagenase or hydroxylamine treatment.

Prior to immunoprobing with the monoclonal antibody, western blots of native particulate transglutaminase run on non-reducing gels were cut into strips, and the strips were then incubated with 0.5 units of either Type VII lipase (from Candida cylindracea) or Type VII collagenase (from Clostridium histolyticum), or 1M hydroxylamine, in 1ml TBS containing 10mM $CaCl_2$, at 37°C for 1 h. As a control, Factor XIII (Boehringer), electrophoresed and western blotted under comparable conditions, was also subjected to enzyme treatment.

These treatments led to the production of a particulate form capable of cross-reacting with the monoclonal antibody (Fig.6.16). Thus fatty acid/glycerol ester cleavage, collagen breakdown or the cleavage of the comparatively rare Asn-Gly bond, reveals the monoclonal epitope. Similar treatment of Factor XIII did not produce a form capable of cross-reactivity with the monoclonal antibody (Fig.6.16).

6.5.2 Carbohydrase treatment.

Western blots of native particulate transglutaminase and Factor XIII, run on non-denaturing gels, were cut into strips and the strips incubated for 1 h at 37°C, with 0.5 units of one of the carbohydrases, mannosidase, glucosidase or galactosidase, in 1ml TBS containing 10mM $CaCl_2$. The plasma transglutaminase Factor XIII was again included as a control.

As expected from previous results, obtained by using lectins, (section 6.4.3.4), mannosidase, galactosidase or glucosidase treatment of western blots did not produce a particulate form capable of cross-reacting with the monoclonal antibody (Fig.6.16). Similarly, carbohydrase treatment of Factor XIII did not lead to the exposure of an epitope for the monoclonal (Fig.6.16).

Figure 6.16 : Enzyme treated western blots of non-denatured particulate tissue transglutaminase, probed with monoclonal anti-guinea pig liver cytosolic transglutaminase.

Samples (~10ug per lane) of particulate and cytosolic (C) tissue transglutaminase, semi-purified (~25%) by anion-exchange, and the plasma transglutaminase Factor XIII, were electrophoresed under non-denaturing conditions on a 7.5% (w/v) polyacrylamide resolving gel (section 2.5.2). Separated proteins were then electoblotted onto nitrocellulose from the polyacrylamiade gel (section 2.5.3.2). Strips of the nitrocellulose, supporting the particulate enzyme (P) and Factor XIII (F), were incubated for 1h at 37^{o}C with one of the following :-

0.5U of Type VII lipase (from Candida cylindracea) (L);

0.5U Type VII collagenase (from Clostridium histolyticum) (Cg);

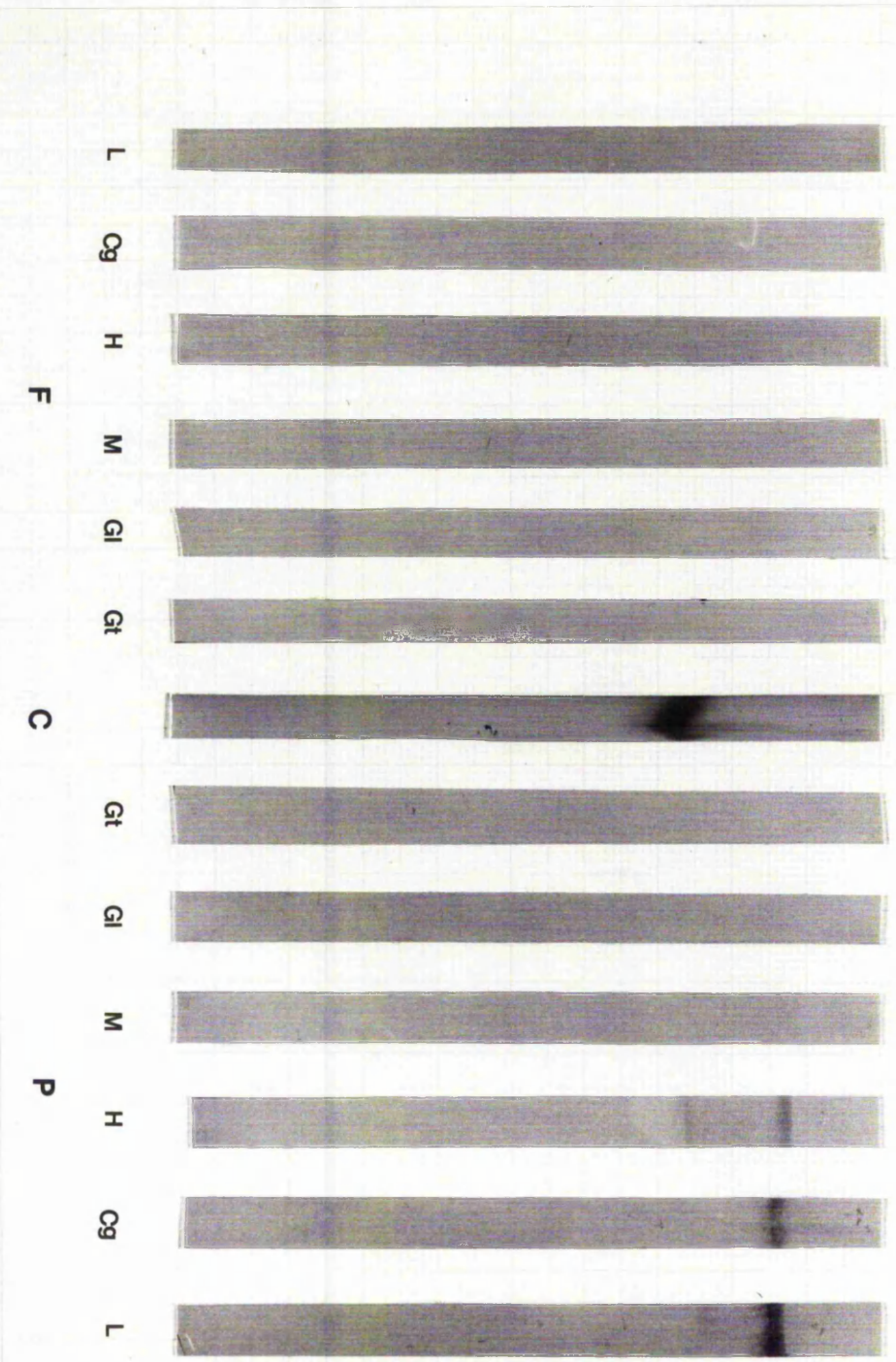
1M hydroxylamine (H);

0.5U mannosidase (M);

0.5U glucosidase (Gl);

0.5U galactosidase (Gt),

in 1ml TBS containing 10mM $CaCl_2$. Western blots were then blocked with 2% (w/v) Marvel in TBS as in section 2.5.4 and probed, for 2h at room temperature, with a 1/100 dilution of mouse monoclonal anti-guinea pig liver transglutaminase antibody in TBS. Blots were developed using biotin conjugated anti-species antibodies (diluted 1/500 in TBS), followed by HRP labelled avidin (diluted 1/5000 in TBS) and chloronapthol reagent as in section 2.5.4.


F
L
Cg
H
M
Gl
Gt
C
Gt
Gl
M
H
Cg
L
P

6.6 Discussion.

The presence of an inactive enzyme in metastatic tumours, but not in normal tissue, begs the questions, what is its cause, and what relationships exist between it and the two active forms present in normal tissue? In order to start to address these questions it was necessary to establish the subcellular localisation of the inactive form, purify each form, and then characterise and compare each form to one another.

The subcellular localisation of the two active forms is already known to the extent that one is predominantly cytosolic and the other is membrane associated. On separation of tumour homogenates into a cytosolic fraction and a 71,000g pellet, approximately 80% of the cytosolic and approximately 70% of the inactive form were found to be located in the cytosolic fraction, with 100% of the particulate being found within the pellet. Thus it can be assumed that the inactive antigen is cytosolic in its localisation.

To purify the three forms, a modification of the method of Connellan et al (1972) was used, followed firstly by immunoaffinity chromatography and secondly by GTP-affinity chromatography. This last step was included initially to ascertain whether or not any of the three forms of transglutaminase were capable of binding to GTP as suggested by various workers (Achyuthan and Greenberg (1987); Bergamini et al (1987) and Bergamini (1988); Lee et al (1989)). Since all three forms bound to the GTP-agarose column, this step was included in the final purification protocol. The final specific activity for the cytosolic enzyme was 7.9 U/ug (after small scale purification) and for the particulate form was 11 U/ug.

Data from experiments undertaken on tumours during growth indicate an inverse relationship between expression of the active cytosolic enzyme and the expression of the inactive antigen, with no apparent correlation with the expression of the particulate enzyme. The presence of the inactive antigen in the cytosol also suggests a link with the cytosolic form of tissue transglutaminase. Since the inactive enzyme protein appears to be larger than either the particulate or the cytosolic enzyme, both by gel filtration chromatography and by SDS PAGE, it is unlikely to be produced by the proteolysis of either form. It is therefore feasible to suggest that the presence of the inactive enzyme protein in the metastatic tumour cell may be due to inappropriate expression of one of the two active forms normally present, probably the cytosolic transglutaminase.

Chung et al (1988) found that it was possible to activate particulate transglutaminase by partial proteolysis and Negi et al (1985) produced an active 50,000 dalton epidermal transglutaminase by partial proteolysis of a large molecular weight epidermal form. Studies involving the limited proteolysis of all three forms gave rise to evidence to further indicate that the inactive form of enzyme protein might be the result of inappropriate expression of the cytosolic enzyme. Treatment of the inactive protein with either trypsin or thrombin led to the appearance of an active form of transglutaminase enzyme displaying a number of biophysical properties comparable to that of the cytosolic enzyme. The major, and active, antigenic product arising from the partial proteolysis of the inactive form co-eluted with the cytosolic transglutaminase form when using both anion-exchange and molecular seive chromatography. No antigen mimicking that of the particulate enzyme was ever detectable in any of these

investigations. Similarly, western blots of non-denaturing gels indicated an electrophoretic pattern for the trypsinised inactive antigen that corresponded to that of the cytosolic transglutaminase.

The active enzyme formed by limited proteolysis is by definition a transglutaminase: it incorporates putrescine into a protein substrate; it is calcium dependent, with a Km for calcium that is similar to that of the cytosolic form; it is heat labile, confirming it as a tissue, rather than an epidermal, transglutaminase; it cross-reacts with both poly- and mono- clonal antibodies raised against the cytosolic form. It is however unable to cross-react with antibodies raised against the a-subunit of Factor XIII suggesting that it is immunologically distinct to this transglutaminase.

Peptide maps produced from tryptic digests of the cytosolic form and the inactive antigen showed approximately 70% homology when immunostained, whilst homology between immunostained peptide maps of the particulate and the inactive produced only 20% homology. Thus it is fairly evident that the cytosolic form of tissue transglutaminase and the inactive antigenic form are closely related. The results also suggest that the inactive form may be a possible precursor of the active cytosolic enzyme.

Similarities also exist between the cytosolic (and therefore the inactive) and the particulate forms of the tissue transglutaminase. The sugars mannose, glucose and galactose do not appear to be present on any form. Polyclonal antibodies raised against the cytosolic enzyme cross-react with the particulate form, and with a greater avidity, as suggested by their relative elution patterns from an anti-cytosolic transglutaminase affinity column. Studies with the monoclonal anti-cytosolic transglutaminase antibody indicate that this antibody does

not cross-react with native particulate transglutaminase, suggesting that the epitope is missing from the protein. However, once denatured by SDS, the particulate enzyme was found to cross-react with this monoclonal antibody, therefore both the cytosolic and particulate enzymes must possess the epitope for this antibody, as does the inactive antigen. Since denaturation of the particulate enzyme reveals the epitope, the suggestion is that in the native enzyme the epitope is masked, either by further post-translational modification as found in epidermal transglutaminase (Chakravarty and Rice, 1989; Rice et al, 1990), by the binding of extraneous material during purification or by differences in the tertiary structure of the two enzymes. Since SDS PAGE indicated each of the two enzymes to have comparable molecular weights, a result contrasting with that obtained from gel filtration chromatography, each of the latter proposals are possible. The observation that treatment with either lipase, collagenase or hydroxylamine, leads to cross-reactivity of non-denatured particulate transglutaminase with the monoclonal antibody suggests that the particulate enzyme associates with both lipids and collagen, and that this association may be responsible for masking the epitope. Previous workers have indicated that tissue transglutaminase has a high affinity for the extracellular matrix (Turner and Lorand, 1989; Achyuthan et al, 1988; Martinez et al, 1989; Conkling et al, 1989), Chakravarty and Rice (1988) showed that myristate and palmitate bound to the epidermal transglutaminase (which is membrane associated) could be released by hydroxylamine, and suggestions have been made that the binding of the cytosolic enzyme to collagen during cell fractionation may account for a number of its particulate properties (Juprelle-Soret et al, 1988). The binding of tissue transglutaminase to specific plasma membrane

domains has also been noted (Tyrrell et al, 1986).

Previous reports (Chang and Chung, 1986) have indicated the molecular weight of the particulate transglutaminase, as calculated by size exclusion chromatography, to be approximately 20,000 daltons greater than the known weight of the cytosolic form, ie. approximately 100,000 daltons. Since separation of proteins by gel filtration is dependent on exclusion by molecular size, any deviations from ideal behaviour, such as the alteration to configuration caused by side chains or associated lipid, will lead to errors in the estimation of molecular weight. SDS-solubilised forms of the particulate and cytosolic transglutaminases, when subjected to SDS PAGE, give molecular weights that are comparable to one another at 87,000 daltons, thus furthering the evidence to suggest that the particulate form may be a post-translationally modified version of the cytosolic enzyme, or vice versa.

7: DISTRIBUTION OF TRANSGLUTAMINASE ACTIVITY AND ANTIGEN IN NORMAL AND MALIGNANT HUMAN BREAST.

7.1 Introduction.

The earlier chapters of this thesis (Chapters 3 and 5) have indicated an apparent inverse correlation between the ability of a tumour to form metastases and the associated level of cytosolic transglutaminase activity. A major cause of the reduction in cytosolic activity, occuring with increasing metastatic potential, is the appearance of an inactive form of the enzyme (Chapter 4). This is an apparently abnormal protein, since its presence is not detectable in normal liver or 'normal' fibroblasts. However, it cannot be ruled out that it is the sensitivity of the current assay that renders its presence in normal tissue undetectable. Similarly, the two non-metastatic tumours assayed, contained no detectable inactive transglutaminase antigen. Hence the presence or absence of inactive transglutaminase antigen could be used as an indication of the metastatic ability of the tumour.

At present, few markers are available that give reliable information as to the presence of distant metastases and therefore the prognosis of the patient. Negative staining of tissue with the antibody KA1 is indicative of a preinvasive or invasive carcinoma (Jarasch et al. 1988); the intensity of tissue staining with the monoclonal antibody NCRC-11 correlates with patient survival (Ellis et al. 1985); the proto-oncogene C-erbB-2 is associated with poor short-term prognosis (Barnes, 1989); and investigations have been carried out using 'epithelial membrane antigen' to detect micrometastases in bone marrow smears, but with the small sample size (1 in 10,000 of total bone marrow) detection by this method is unlikely before clinical detection (Dearnley et al. 1981). With a choice of therapies now

available. it is an obvious advantage to have accurate information as to the likelihood of distant invasion so as to select the most appropriate therapy regime. If a panel of markers could be utilised, the prognosis would potentially be more accurate and reliable.

To ascertain the usefulness of transglutaminase as a marker in human breast cancer, a number of biopsy samples (22 tumours and 3 'normals') were assayed blind for their transglutaminase antigen and activity profiles. 'Grades' were applied to the tumours to denote their metastatic potential, the level of the grade being dependent on the ratio of inactive enzyme to cytosolic enzyme. The results obtained were then compared to the histological grades and pathologist's report in an attempt to correlate with prognosis.

7.2 Measurement and distribution of transglutaminase activity and antigen.

The first step towards evaluating transglutaminase as a tumour 'malignancy marker' was to demonstrate the presence of the enzyme, and its distribution, in normal breast tissue and carcinoma of the breast. Accordingly, tissue samples were homogenised, detergent extracted and the extracts separated by anion-exchange chromatography. Eluent fractions were then assayed for transglutaminase activity, by the Ca^{2+} dependent incorporation of radiolabelled putrescine into N,N'-dimethylcasein, and for antigenic transglutaminase by the quantitative sandwich ELISA using the normal assay (section 2.6.3.1) for assessment of particulate antigen expression and a modification of the assay, whereby a mouse monoclonal anti-cytosolic transglutaminase antibody at a 1/1000 dilution was used in place of the rabbit anti-rlct antibody for assessment of cytosolic and inactive antigen expression. The monoclonal anti-cytosolic transglutaminase antibody was used to quantitate inactive and cytosolic transglutaminase levels as this antibody crossreacts with the two forms equally enabling the concentration of the inactive form to be calculated directly from a standard curve of cytosolic transglutaminase (Fig.7.1).

From these studies it was apparent that tissue transglutaminase was present in both normal and abnormal breast tissue. But it was only in abnormal breast tissue that there was any evidence for the presence of an inactive antigenic form (Table 7.1) (Fig.7.2a). Two active forms of tissue transglutaminase were detectable by the sensitive ^{3}H-putrescine assay (Fig.7.2b) but activity levels were too low for accurate estimation using the ^{14}C-putrescine assay.

Figure 7.1 : Standard curve for quantitative sandwich ELISA using monoclonal anti-guinea pig cytosolic transglutaminase.

Triplicate dilutions (lug - 250pg) of guinea pig liver cytosolic transglutaminase and rat P8 sarcoma inactive transglutaminase were introduced into the optimised assay (section 2.6.3.1).

Solid lines indicate the region of equivalent linearity.

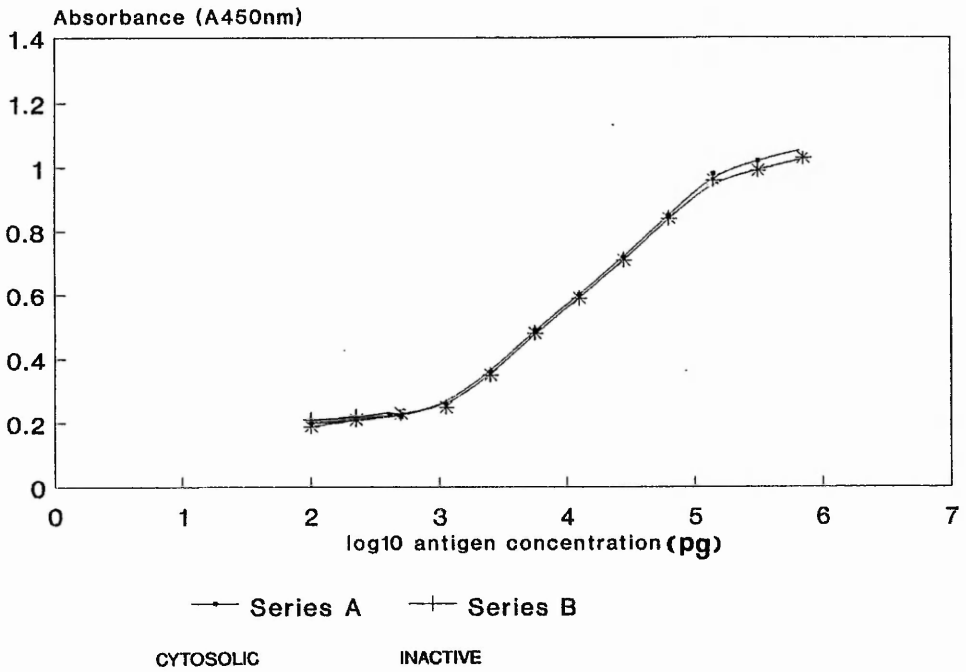
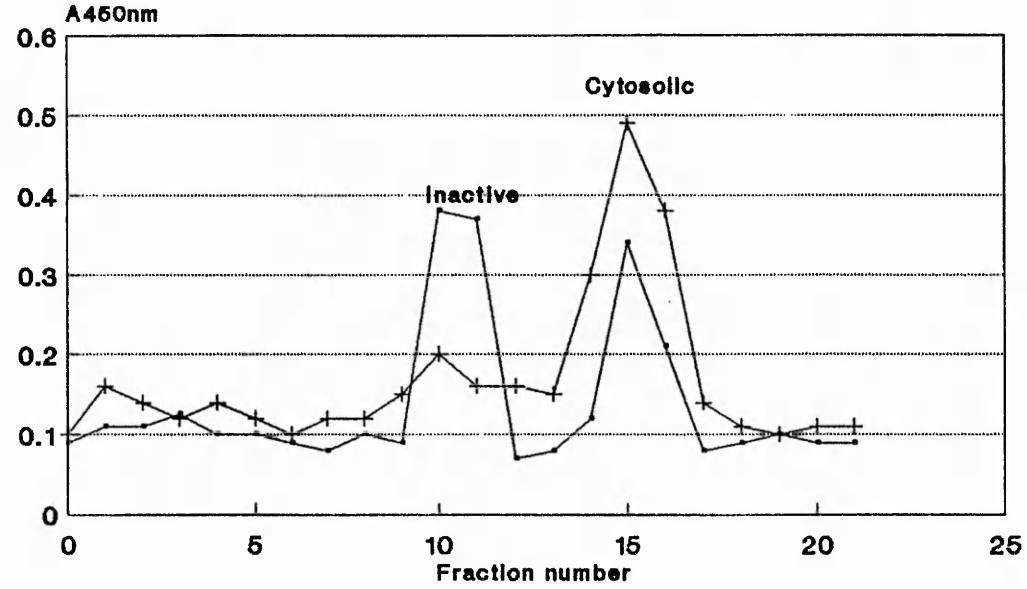

Absorbance (A450nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
7
log10 antigen concentration (pg)
Series A
Series B
CYTOSOLIC
INACTIVE

Fig. 7.2 : Typical anion-exchange chromatography elution profiles for active and antigenic transglutaminase from breast carcinoma tissue and normal breast tissue.

a) Antigen profiles were obtained using a poly-l-lysine ELISA, as described in the legend to Table 7.1.

b) Activity profiles were obtained using the ^{3}H-putrescine assay, as described in the legend to Table 7.1.


A450nm
0.6
0.5
0.4
0.3
0.2
0.1
0
Cytosolic
Inactive
0
5
10
15
20
25
Fraction number
Series A
Series B
Tumour
Normal

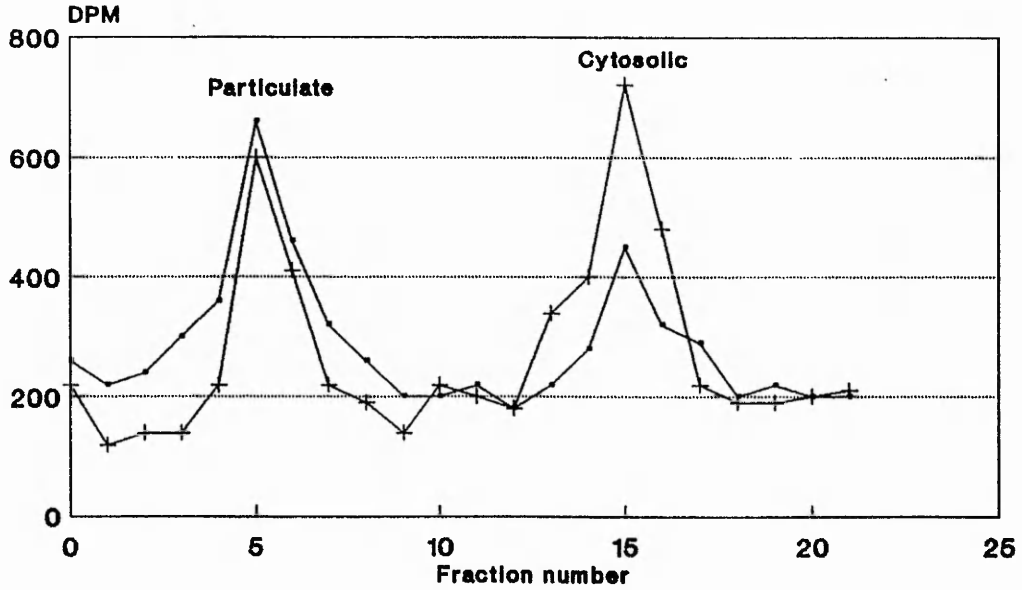

DPM
800
600
400
200
0
Particulate
Cytosolic
0
5
10
15
20
25
Fraction number
Series A
Series B
Tumour
Normal

Table 7.1 : Antigenic transglutaminase levels in human breast tumour and normal human breast tissue.

All samples were snap frozen in liquid nitrogen within 10min of resection and transported in liquid nitrogen vapour.

Samples 1 - 17 were obtained from The City Hospital, Nottingham, stored at -120°C, and assayed within 10 days post-operative.

Samples 18 - 22 were obtained from The Royal Hallamshire Hospital, Sheffield, and were stored at -120°C.

Sample size ranged from 100 - 800 mg wet weight of tissue.

Wherever possible, 200mg of tissue was homogenised to give a final 20% (w/v) homogenate (section 2.4.1). Protein loaded onto the Mono-Q column (1ml) ranged from 3 -5 mg, equivalent to 100 - 220 ng transglutaminase antigen. Fractions, 500ul, were collected; 100ul samples from each fraction were assayed in the poly-l-lysine ELISA (section 2.6.3.2) to give antigen elution profiles. 100ul samples from the pooled particulate peaks were assayed in the quantitative ELISA (section 2.6.3.1) and 100ul samples from pooled inactive and cytosolic peaks were assayed in the quantitative ELISA with the monoclonal anti-cytosolic transglutaminase antibody in the place of the rabbit anti-rlct polyclonal antibody; 24ul samples were assayed for transglutaminase activity in the ^{3}H-putrescine assay (section 2.6.1.2) to give activity elution profiles.

ND refers to 'not detectable'.

N refers to a 'normal' tissue sample.

Identity number	Antigen levels ng/ug DNA		
	Particulate	Inactive	Cytosolic
1		2.8	3.2
2		4.5	3.3
3	4.2	5.0	5.4
4	2.3	3.6	4.1
5	2.6	3.1	2.0
6	3.0	4.4	2.6
7	2.5	2.7	2.4
8	2.6	3.0	2.7
9	2.6	3.1	2.4
10	3.2	3.0	2.6
11	2.8	2.5	4.2
12	2.7	2.8	3.7
13	2.8	3.2	2.6
14	2.5	2.7	3.3
15	1.9	4.1	3.7
16	2.2	3.4	2.4
17	2.4	3.5	3.9
18	2.3	2.8	3.4
19	3.7	4.9	3.7
20	2.9	2.6	3.4
21	3.3	3.2	3.9
22	2.0	3.8	3.3
3 N	3.1	ND	9.8
7 N	1.9	0.9	5.1
11 N	2.4	ND	5.8

Cytosolic transglutaminase antigen levels were higher in normal breast than in the abnormal sample from the same patient; with particulate levels remaining constant between the two. However, with the low number of normal samples available, and the wide spread of data for the carcinomas (2 - 5.4 ng transglutaminase / ug DNA for the cytosolic and 1.9 - 4.2 ng transglutaminase / ug DNA for the particulate) it is impossible to make any definitive statement about 'normal' and 'abnormal' levels.

Immunohistochemistry (IHC) was also used in an attempt to establish the physical location of the enzymes in tissue (Fig. 7.3). Unfortunately, the monoclonal anti-cytosolic antibody is required to be used neat in IHC, thus its use was precluded in these studies since this would have greatly reduced our limited stocks. Thus it is impossible to distinguish between the three forms. However, valuable information was gained in the optimisation of techniques required for future IHC studies. Acetone treated, paraffin embedded sections of breast tumour tissue were succesfully immunostained with a 1/1000 dilution of rabbit anti-rat liver cytosolic transglutaminase antibody. Haematoxylin was used to counterstain the sections. Full details of the staining protocol are given in the legend to Fig.7.3.

Staining tended to be along the periphery of cell groups, and was not restricted to the cytosol of a few cells, as was found by Fesus (personal communication) and Piacentini, 1990. However, both these groups of workers used a monoclonal antibody to cytosolic transglutaminase as the immunoprobe, and this may not crossreact with the particulate form. The particulate staining seen in this study may be due to association of the cytosolic enzyme with membrane, or extracellular, proteins during sectioning, or may be due to the

presence of the particulate form. There was no apparent correlation between the level of transglutaminase staining and the malignancy of the tumour. This is to be expected in this study, as the antibody used crossreacts with all three forms of tissue transglutaminase present, and the total amount of antigenic transglutaminase does not correlate with the aggresiveness of the tumour.

Figure 7.3 : Photomicrographs of breast tumour tissue stained immunochemically with rabbit anti-rat liver cytosolic transglutaminase.

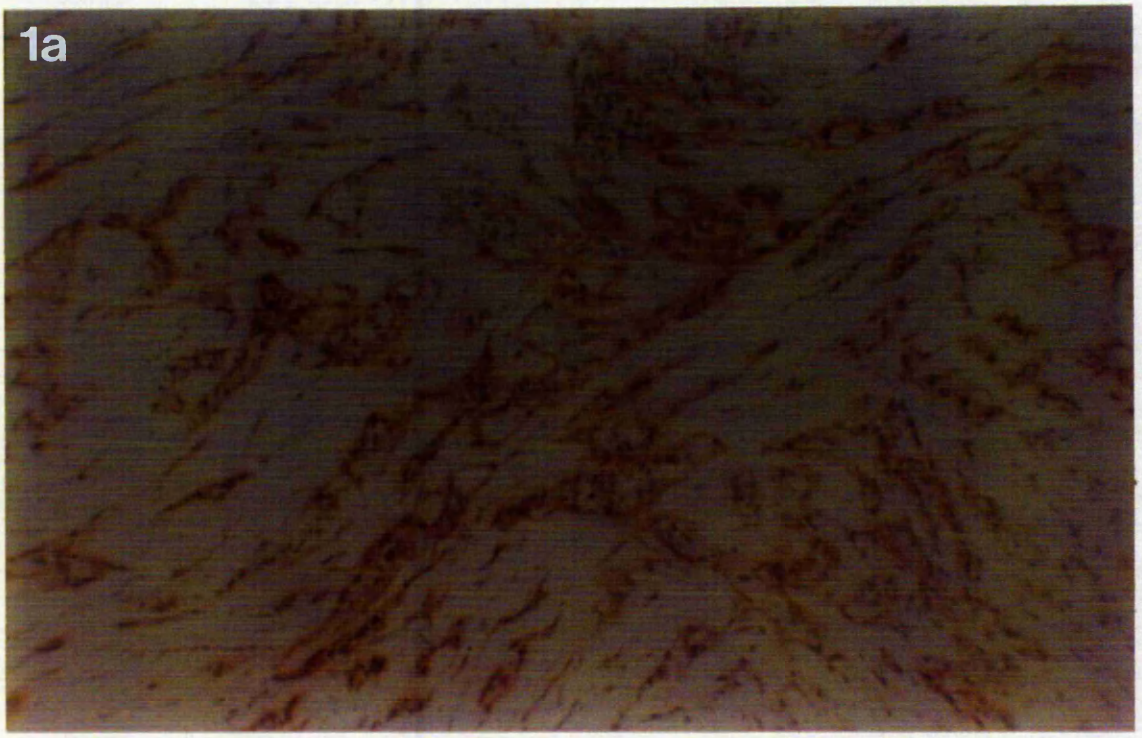
Paraffin embedded sections of breast tumour tissue were acetone treated for 10min. Endogenous peroxidase activity was blocked by immersion in 0.3% (v/v) hydrogen peroxide in methanol for 20min. and the sections were then washed, first in water and then in TBS. Sections were then immersed for 30min in rabbit anti-rat liver cytosolic transglutaminase diluted 1/1000 in normal swine serum / TBS (1:5). After three washes in TBS, anti-rabbit-HRP conjugate (1/1000 dilution) was applied for 30min. Sections were again washed in TBS, developed for 5min using DAB solution and washed in deionised water. After treating the sections with copper sulphate solution (0.5% (w/v) $CuSO_4$ in 0.8% (w/v) NaCl) for 5min and washing in water, sections were counterstained with haematoxylin.

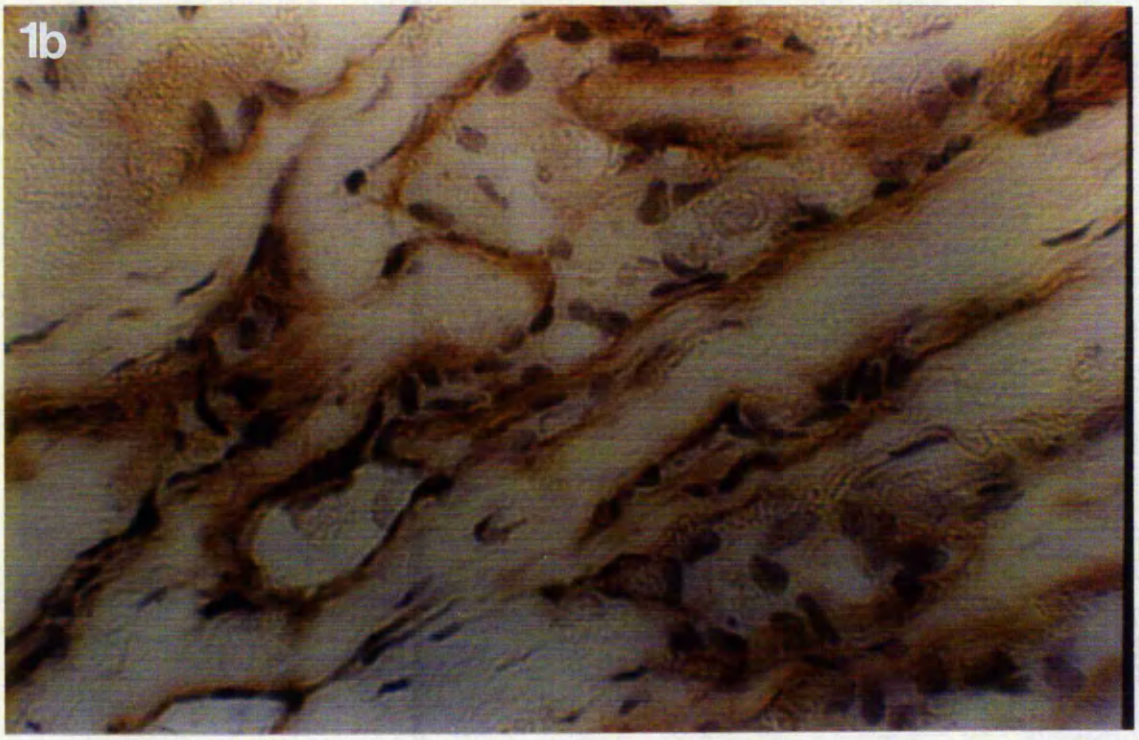
Metastasised tumour stained with :-

1: immune serum, x250, x1000.

2: pre-immune serum, x250.

Sectioning and staining was performed by technical staff in the Histopathology department of The City Hospital, Nottingham.


1a


1b


2

7.3 Diagnostic grading.

Histological assessment of the degree of differentiation of a tumour has been, and still is, used as a grading system for malignancy. The initial assumption was that the less differentiated the tumour cells became, the more malignant the tumour. It is now appreciated that this is an oversimplification, and although histological staging gives a fairly accurate index of malignancy and prognosis for some carcinomas such as cervix, colon and thyroid, for others it is a very inaccurate means of assessing malignancy. Tumours may be further described by their stage, this being the definition of the extent of tumour growth and progression at one point in time. Staging may be done clinically, by physical examination, X-ray and endoscopic examination; radiographically, by tomography, lymphangiography and radioisotope scanning; surgically, by direct surgical exploration; or pathologically, by the use of biopsy procedures. The method of staging used is dependent on the type of cancer being staged.

To assess the potential of transglutaminases as 'malignancy markers', histological grades and pathological reports were compared to a biochemical grading based on the ratio of inactive antigenic transglutaminase to cytosolic transglutaminase antigen. The monoclonal anti-cytosolic transglutaminase antibody was used to quantitate inactive and cytosolic transglutaminase levels as this antibody crossreacts with the two forms equally enabling the concentration of the inactive form to be calculated directly from a standard curve of cytosolic transglutaminase (Fig.7.1).

Reference to Table 7.2 indicates a positive correlation between the biochemical grading of a tumour and its extent of invasion

as assessed pathologically. Tumours graded A, indicating high metastatic potential biochemically, tended to show clinical signs of invasion, either lymphatic or vascular, whilst fewer clinical signs of invasion were noted with those tumours graded B or C. Of those tumours graded, 75% of the biochemical grades and 45% of the histological grades accurately predicted the presence or absence of metastases or invasion of circulatory systems. For the biochemical grades, 18% of the tumours gave false positives (however in 2 of the 4 cases no lymph nodes were examined and it is therefore not possible to say whether or not local invasion had occurred) and 9% gave false negatives in as much as they were indicated to be only weakly metastatic but had in fact metastasised. With the histological grading system, 40% of the tumours graded gave false positives (of which in only 1 of the 6 cases were no nodes had been sampled) and 14% gave false negatives. It is of course possible with all of the false positives that distant metastases had occurred and remained undetected.

Table 7.2 : Tumour grades and pathological findings.

Histological grading

nuclear morphology :-	regular size and staining	1
	moderate pleomorphism	2
	marked pleomorphism	3
mitotic figures :-	< 1 per high power field	1
	1 - 2 per high power field	2
	> 3 per high power field	3
tubule formation :-	well marked with acinar arrangements	1
	moderate	2
	little or none, cells in sheets or strands	3

Grade 1 :-	score 3 - 5	well differentiated , good prognosis?
Grade 2 :-	score 6 - 7	moderate differentiation, moderate
Grade 3 :-	score 8 - 9	poorly differentiated, poor prognosis?

Biochemical grading : using ratio of inactive transglutaminase to cytosolic transglutaminase. (I : C)

1 : < 1	A	highly metastatic
1 : 1 - 1.5	B	weakly metastatic
1 : 1.5 - 3.0	C	non-metastatic

Pathological findings are taken from the pathologist's report for each patient.

ID N°	Hist. grade	Biochem. grade	Pathological findings.
1	1 (2.2.1)	B (1:1.14)	Ductal carcinoma. in situ Tubular carcinoma. infiltrating Internal mammary node is free.
2	1 (2.2.1)	A (1:0.75)	Ductal carcinoma Tumour present in all nodes taken.
3	3	B (1:1.1)	Lobular carcinoma no evidence of malignancy.
4	2	B (1:1.1)	Ductal carcinoma. infiltrating No vascular invasion Nodes sampled were free.
5		A (1:0.65)	Ductal adenocarcinoma Vascular invasion No nodes sampled.
6	2	A (1:0.6)	Ductal carcinoma Nodes sampled were free.
7		A (1:0.9)	Infiltrating carcinoma No vascular invasion No lymph nodes sampled.
8	1 (1.1.1)	A (1:0.9)	Infiltrating adenocarcinoma Lymph node sampled is free Perineural lymphatic invasion.
9	2	A (1:0.85)	Lobular carcinoma Tumour present in all nodes taken.
10	2	A (1:0.9)	Lobular carcinoma. infiltrating No nodes sampled.

ID N°	Hist. grade	Biochem. grade	Pathological findings.
11	2 (3.2.1)	C (1:1.8)	Ductal adenocarcinoma Nodes sampled were free.
12		B (1:1.28)	Phyllodes tumour, benign.
13	2	A (1:0.8)	Infiltrating carcinoma Apical lymph node free.
14		B (1:1.2)	Ductal carcinoma, infiltrating Mammary node clear.
15	2 (3.2.1)	A (1:0.9)	Invasive ductal carcinoma 2/3 nodes taken contain tumour.
16	3 (3.3.3)	A (1:0.7)	Invasive ductal carcinoma All 10 nodes taken contain tumour.
17		B (1:1.1)	Carcinoma Lymph nodes taken contain tumour.
18		B (1:1.23)	Adenocarcinoma All nodes taken were free.
19	2	A (1:0.8)	Ductal adenocarcinoma 3/9 nodes taken contain tumour Vascular invasion.
20		B (1:1.29)	Adenocarcinoma Poorly differentiated No evidence of invasion.
21		B (1:1.2)	3/12 nodes taken contain tumour.
22	2	B (1:1)	Infiltrating carcinoma No nodal involvement.

7.4 Discussion.

The use of tumour markers, both biochemically and immunohistochemically, has expanded rapidly over the last few years. Many markers are used routinely to aid in the diagnosis of a particular specimen.

Neither the staging nor the grading of a tumour provide unequivocal evidence as to it's exact progression, however they are useful aids to the oncologist in the selection of the most appropriate therapy regime, and they give a general guide to the patients prognosis. One of the biggest imponderables in tumour staging and grading is the presence of micrometastases in lymph nodes or distant organs, undetectable by current procedures, that mean a potentially fatal event has already occurred, even though minimal involvement may have been indicated. It is here that a histological or biochemical 'marker' for malignancy would come into its own, ideally answering the question as to whether or not a tumour has metastasised and at worst aiding the grading of a tumour by defining that tumours metastatic potential.

From the data presented, it is clear that there is a potential for the use of transglutaminases in the estimation of malignancy. However, the assay in its present state is time consuming, taking the best part of two days from receipt of sample to final result; potentially hazardous with the possible release of viruses; and expensive equipment not normally available in histopathology laboratories is required. The type of marker detection most easily included in routine histopathological examination of tumour biopsys would be immunohistochemical. The ability to stain transglutaminase in

sections may therefore provide the answer to simplification of the assay. Development of antibodies specific for each form of transglutaminase would allow for quick and easy subjective evaluation of inactive to cytosolic ratios, but would require standardisation between users. By increasing the sensitivity of the assay with more specific antibodies it might also be possible to achieve a reliable, non-subjective, quantitative result from homogenates of tumour aspirates by use of an ELISA method.

Another possible marker of metastatic potential related to transglutamianse activity in tumours, is the apoptotic index. This is similar to the mitotic index commonly used in histological grading in that it relates to the rate of growth of a tumour, but instead of giving a value for the rate of increase of cell number, it gives a value to the rate of programmed cell deletion. Recent evidence has linked transglutaminase activity to the events involved in the process of programmed cell death or 'apoptosis' (Fesus et al, 1987, 1989). Data presented in sections 3.2.2 and 3.4.1 further indicated a correlation between cytosolic transglutaminase activity and apoptosis with decreases in cytosolic activity reflected by decreases in the apoptotic index. Assessment of the apoptotic index, in a manner similar to that used already for the mitotic index, could therefore add to the description of a tumour in two ways. Firstly, since apoptosis balances the process of cell proliferation in tissue homeostasis, the apoptotic index would give a further parameter by which to define the growth rate of a tumour. Secondly, because of the correlation with cytosolic transglutaminase activity, the apoptotic index could be used to give a histological estimate of malignancy, in that a high index would indicate high cytosolic activity and therefore a low metastatic

potential. with a low index indicating a high metastatic potential. The scoring of apoptotic cells in histological sections would be easy to include in a routine histopathological assessment. and could greatly improve the evaluation of prognosis. however. the definition of a 'high' or 'low' index is as yet arbitrary as no 'normal' values are available and so a comparison with normal breast tissue from each patient would be necessary.

8 GENERAL DISCUSSION.

Since the observation, by Laki et al, 1966, of tissue transglutaminase activity in the YPC-1 mouse tumour, numerous investigations into the association of tissue transglutaminase with the process of carcinogenesis have been reported (section 1.3.5). Such studies provide valuable information both about the carcinogenic process and the intracellular role, or roles, of tissue transglutaminase. The level of transglutaminase activity observed in neoplastic tissues tends to be far lower than in their comparable normal counterparts (Birckbichler et al, 1976a; Birckbichler et al, 1977; Birckbichler et al, 1978; Berntorp et al, 1984; Barnes et al, 1985; Hand et al, 1987) and if, as has been speculated (see chapter 1), transglutaminase plays a fundamental role in controlled cellular functioning then such a reduction could be involved in the disorganisation of cellular mechanisms characteristic of neoplasia. The observation that two polyclonal antibodies, raised against cytosolic transglutaminase from guinea-pig liver and rat liver respectively, show comparable affinity for cytosolic transglutaminase from hamster fibrosarcomas and from guinea-pig liver (section 4.4.3), and the high degree of similarity between tissue transglutaminase genes from different species (section 1.4), suggests that cytosolic tissue transglutaminase is a highly conserved protein, further indicating the importance of this enzyme in cellular function. If reductions in activity occur concomitently with the onset of malignancy, either causally or as a product of transformation, then the potential exists for the use of probes to transglutaminase as markers of malignancy. Alternatively, if such a reduction was causative in the development of

the malignant phenotype, methods of reversing, or preventing, the depletion of active transglutaminase might prove curative.

In cultured metastatic variants of an HSV-2 induced hamster fibrosarcoma and the B16 mouse melanoma, the level of total tissue transglutaminase activity was found to correlate inversely ($r = -0.835$ and $r = -0.996$ respectively) with the metastatic potential of the cell line 'in vivo' (Griffin et al, 1989; Knight et al, 1990a, 1990b); whilst the correlation between the invasive capacity 'in vitro' of the HSV-2 variants and transglutaminase activity, although inverse, was weak ($r = -0.5$). This suggests that any involvement that translgutaminase may have in the establishment of secondary foci is associated with the survival of cells in the circulation, and / or extravasation, rather than the invasion and intravasation of cells. Further evidence for this comes from the behaviour of the highly metastatic Met F cell line which displays, as far as transglutaminase is concerned, a weakly metastatic phenotype 'in vitro' but has a high 'in vitro' invasive capacity, suggesting that a low level of transglutaminase activity is not a trigger or a requirement for invasion. This conclusion is also supported by the work of Delcros et al, 1986, who first noted an inverse relationship between transglutaminase activity and the metastatic ability of cells. In this system, cells from variant sublines of a rat rhabdomyosarcoma were injected intravenously, and so the initial intravasation step was accomplished artificially, therefore indicating that any role played by transglutaminase in metastasis must be after the intravasation stage. Interestingly, Edward and MacKie, 1989, found that pre-treatment of the mouse melanoma cell line B16BL6 (a highly metastatic subline derived from a bladder metastasis of the B16 melanoma line) with all-trans

retinoic acid, reduced the number of pulmonary metastases produced after intravenous injection. Retinoids have also been shown to inhibit the formation of lung colonies by B16 melanoma cells (Lotan and Nicholson, 1981; Edward et al, 1989), human breast carcinoma cells (Fraker et al, 1984) and squamous carcinoma cells (Couch et al, 1988), and influence cell-cell and cell-substratum adhesion (Bertram et al, 1981; Kamei, 1983). Many investigators have studied the induction of tissue transglutaminase activity and expression caused by retinoic acid (see section 1.5), with the initial studies being carried out on melanoma cells, when Scott et al, 1982, noted that retinoic acid caused the induction of tissue transglutaminase activity. Thus decreases in the number of metastases observed after retinoic acid treatment might be due to some effect of the concomitant increase in transglutaminase activity within the cells, since the survival of tumour cells in the circulation, and / or their extravasation, appears to be inversely related to transglutaminase activity. The mechanism whereby retinoids inhibit metastasis is not clear, although the modulation of cell adhesion, via cell-cell and cell-substratum adhesion, has been suggested. The potential exists for transglutaminase to influence cell-cell and cell-substratum adhesion (section 1.3.3), and so the proposed modulation of tumour cell adhesion by retinoids, leading to inhibition of the processes involved in extravasation, might be mediated by alterations to tissue transglutaminase levels.

Previous workers have suggested that the loss of activity associated with neoplasia is brought about by a 'redistribution', from the cytosol to the particulate fraction, of tissue transglutaminase activity (Birckbichler et al, 1976; Barnes et al, 1985). Following a report, by Chang and Chung, 1986, of the existence of two forms of

tissue transglutaminase in liver, a cytosolic and a particulate, Hand et al, 1988, reported that the decrease in total transglutaminase activity observed in chemically induced rat liver hepatocellular carcinomas was due to a selective reduction in the activity of the cytosolic form of the enzyme rather than a translocation of enzyme from the cytosol to the membrane. Assessment of the subcellular distribution of transglutaminase activity in cultured hamster fibrosarcoma variants and mouse melanoma variants indicated that the differences in total activity observed between cell lines were again due solely to alterations in the amount of cytosolic activity present. It is therefore more appropriate to say that cytosolic transglutaminase activity and metastatic potential are inversely related.

In epidermis, the action of epidermal transglutaminase is thought to be involved in the formation of the insoluble keratinocyte envelope which is heavily crosslinked by the dipeptide $\epsilon(\gamma$-glutamyl) lysine (section 1.2). During programmed cell death, detergent insoluble 'envelopes', similar to the keratinocyte envelope, termed apoptotic bodies are formed (section 1.3.4). One of the products of the action of tissue transglutaminase in a number of cell types is a highly crosslinked heavy molecular weight 'polymer'. In liver, Fesus et al, 1989, suggested these $\epsilon(\gamma$-glutamyl)lysine crosslinked 'polymers' were the apoptotic bodies formed during programmed cell death and that transglutaminase was involved in their formation. By allocating an apoptotic index, determined by the percentage of detergent insoluble apoptotic bodies isolated from a given number of cultured cells, to each variant cell line a further parameter for the description of the cell line was obtained. Again, this parameter was inversely related to metastatic potential and correlated directly with the amount of

cytosolic activity present in the cell line, suggesting that it is the cytosolic form of tissue transglutaminase that is involved in the formation of the apoptotic envelope during programmed cell death. This supports the work of Thacher and Levitt, 1987, who demonstrated the Ca^{2+} induced formation of cross-linked envelopes in lung cell lines where a soluble transglutaminase, and not the epidermal transglutaminase, was expressed; and Schmidt et al, 1988, where non-competent transformed keratinocytes, devoid of epidermal transglutaminase but containing a cytosolic transglutaminase, were shown to be capable of forming envelope-like structures. Recently, Piacentini, 1990, has reported a connection between the level of expressed tissue transglutaminase in a tumour and its apoptotic rate, he also noted that stimulation of transglutaminase expression by retinoic acid led to an increase in the apoptotic index, whilst reduction of transglutaminase by DFMO was paralleled by a reduction in apoptosis. However, in murine keratinocytes, TGF-β has been shown to eliminate calcium induced terminal differentiation despite the induction of epidermal transglutaminase activity by the calcium stimulus (Reiss and Zhou, 1989). Therefore, in the epidermal system, induction of transglutaminase activity does not of necessity lead to the formation of an envelope matrix.

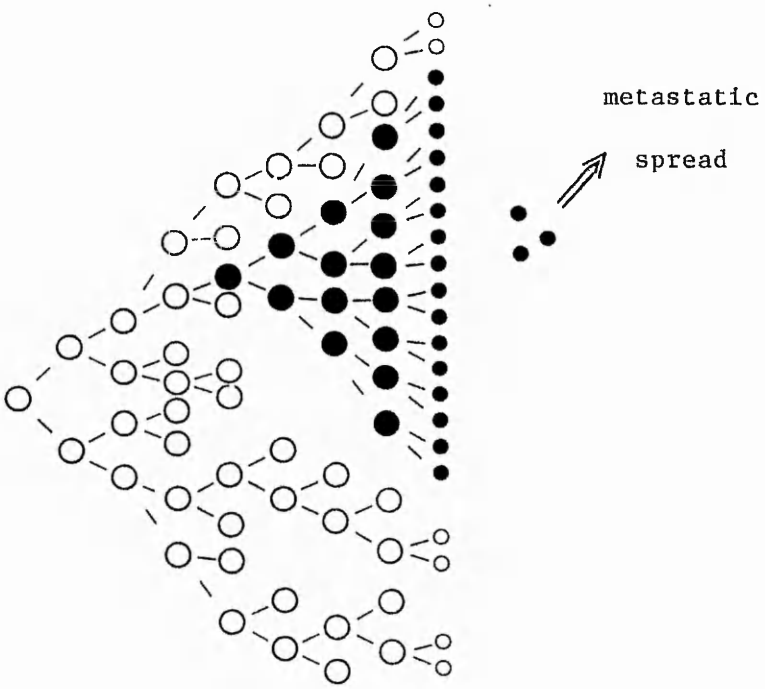
It is, however, invalid to assume that the 'in vitro' expression of a parameter of cellular function should be equivalent to its 'in vivo' expression, since it is as yet impossible to mimic the complex interactions of cells, tissues and hormones that characterise 'in vivo' growth. This is exemplified by the differences in the level of expression of transglutaminase activity 'in vitro' and 'in vivo', where tumour cells are open to the influence of the environmental

pressures exerted by the host. Only the highly metastatic cell line Met F, which did not behave as expected 'in vitro', showed no significant difference between its 'in vitro' and 'in vivo' levels, with the other fibrosarcoma lines tested exhibiting increased activity 'in vivo'. Results obtained from subcutaneously grown solid tumours confirmed the inverse relationship between cytosolic transglutaminase activity and metastatic potential originally observed in cultured cell lines (Griffin et al, 1989; Knight et al, 1990a). The observations of previous workers, who noted that total transglutaminase activity decreased with the progressive growth of metastatic tumours (Barnes et al, 1985; Hand et al, 1988) were also confirmed. The highly metastatic tumours, in both the hamster and rat sarcoma systems, progressively lost cytosolic transglutaminase activity during their growth, whilst in the weakly metastatic tumours, although fluctuations were observed in the level of cytosolic activity, no significant progressive decrease occurred during the time period studied. However, extrapolation of the rate of decrease of cytosolic activity in the moderately metastatic parent hamster fibrosarcoma tumours suggests that decreases as significant as those seen in the highly metastatic tumours might occur by day 60 of growth.

Clonal expansion of heterogenous cell populations occurs during the growth of a tumour, with variant progeny arising from clones due to genetic instability. Those cells with a selective advantage proliferate and become dominant, providing a stepwise sequence of evolution producing evermore abnormal and aggressive cells (Fig. 8.1). If those cells containing low levels of active cytosolic transglutaminase, or possibly no active cytosolic enzyme, were to have a growth advantage over other cells in the tumour population, then

Figure 8.1 : Model of clonal evolution in neoplasia.

Schematic representation of clonal dominance of primary tumours by subpopulations having a selective growth advantage. Comparison of 'late' stage primary tumour to metastases may therefore show little appreciable difference, whilst 'early' stage tumours may vary considerably from metastases.



their clonal expansion would lead to an overall decrease in the level of active cytosolic enzyme in the tumour, as is observed in the highly metastatic tumour lines. The hypothesis that only those cells undergoing programmed cell death express active transglutaminase, a theory proposed by Fesus and co-workers (Fesus et al, 1987; Piacentini 1990; Fesus, personal communication, 1990), can now be extended to read 'only those cells undergoing programmed cell death express active cytosolic transglutaminase' thus discriminating between the two forms of the enzyme. By combining the apoptotic index and the level of cytosolic transglutaminase activity, and working on the above hypothesis, it is possible to estimate the level of cytosolic activity per apoptotic cell for each of the hamster fibrosarcoma and mouse melanoma cell lines (Table 8.1). The figures obtained (U/apoptotic cell) suggest that every cell undergoing programmed cell death, regardless of the cell subline of origin, expresses an equivalent amount of cytosolic transglutaminase activity. Using U/apoptotic cell, it is then possible to estimate the number of cells undergoing apoptosis within a tumour at any stage of its growth (Table 8.2). From these estimates it is possible to suggest that in the highly metastatic tumours, clonal expansion of cells unable to undergo programmed cell death is occurring as the number of apoptotic cells decreases. It is also interesting to note that if T_0 is included (ie. the inoculum of cells) then there is an initial rise in the number of cells undergoing programmed cell death within the tumour mass (Fig. 8.2). This could be the result of attack by the hosts immune system, as it has been proposed that NK cells mediate cell death by inducing apoptosis and therefore, under the terms of the hypothesis, inducing cytosolic transglutaminase activity, this would then cause the difference seen

Table 8.1 : Estimated cytosolic activity per apoptotic cell 'in vitro'.

Cell line	Metastatic potential	U/apoptotic cell
BHK	0	0.0138
Met D	1/20	0.0136
Met C	4/20	0.0141
Parent	6/19	0.0144
Met B	20/20	0.0121
Met E	19/19	0.0130
Met F	20/20	0.0145

Cytosolic activity per apoptotic cell was calculated using the apoptotic indices given in Table 3.7 and the cytosolic activity values given in Table 3.4, and is based on the hypothesis that only those cells undergoing programmed cell death express cytosolic transglutaminase activity.

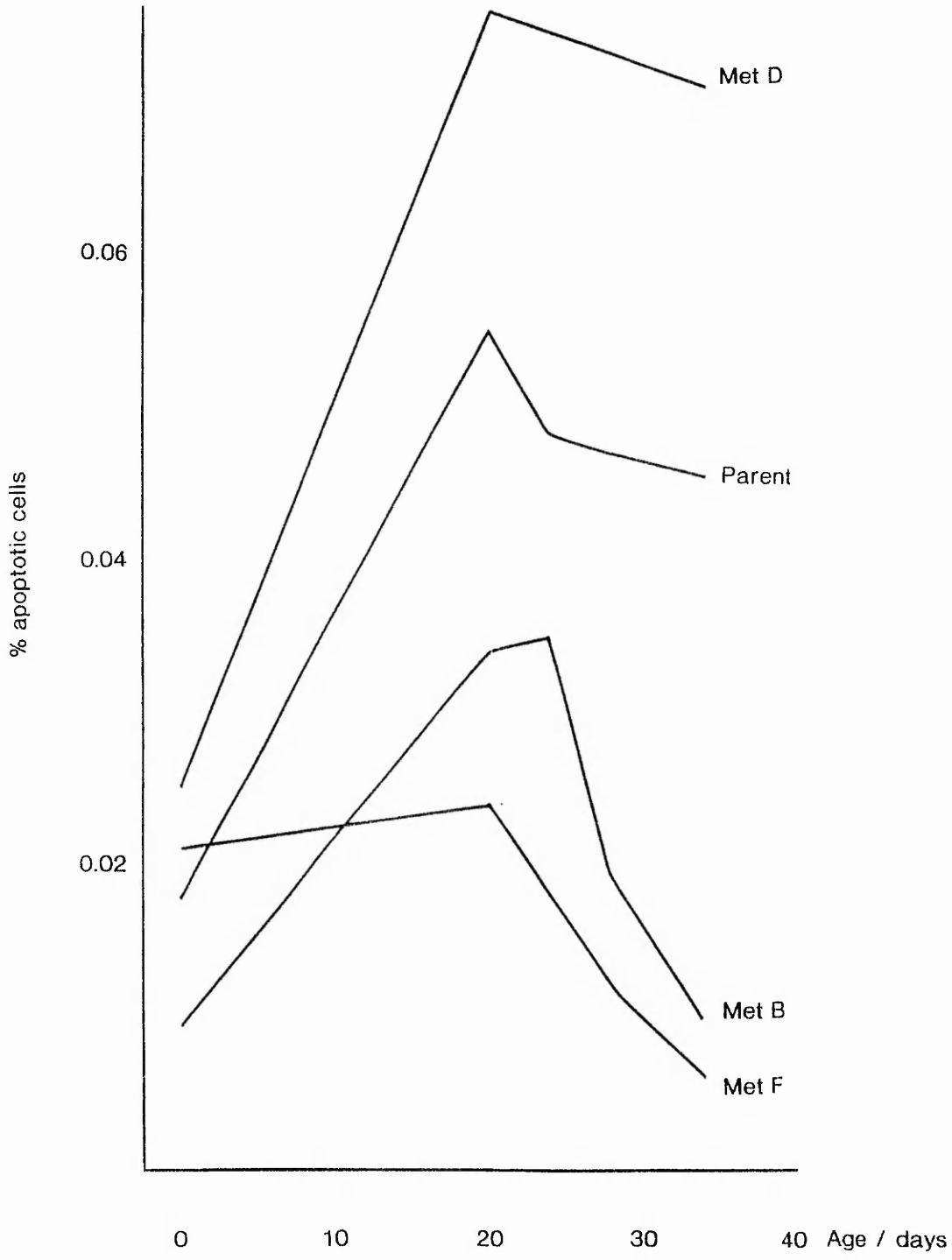
Table 8.2 : Estimation of the percentage of cells within a tumour that are undergoing apoptosis.

Variant	% of cells undergoing apoptosis			
	Day 20	Day 24	Day 28	Day 34
Met D (1/20)	0.076			0.071
Parent (6/19)	0.055	0.048	0.047	0.043
Met B (20/20)	0.034	0.035	0.019	0.010
Met E (19/19)	0.024			0.007
Met F (20/20)	0.024	0.018	0.012	0.006

By using the estimate of the cytosolic activity per apoptotic cell (table 8.1) and the level of cytosolic activity within a tumour, it is possible to calculate the number of apoptotic cells per mg DNA within the tumour ($\frac{\text{U/mg DNA}}{\text{U/ apoptotic cell}}$). The number of cells per mg of DNA can be calculated from the transglutaminase activities given in Table 3.4 ($\frac{\text{U/mg DNA}}{\text{U/}10^6 \text{ cells}}$). From these figures it is then possible to calculate a figure for the percentage of cells within a tumour that are undergoing apoptosis ($\frac{\text{no. apoptotic cells per mg DNA}}{\text{no. of cells per mg DNA}} \times 100$).

Figure 8.2 : Percentage of cells within a tumour undergoing 'apoptosis' during tumour growth.

The percentage of cells undergoing programmed cell death within the cell inoculum is taken from Table 3.7. The percentage of cells undergoing programmed cell death within the tumour at the given days of growth was calculated as detailed in the legend to Table 8.2.


Met D
Parent
Met B
Met F
0.06
0.04
0.02
% apoptotic cells
0
10
20
30
40
Age / days

between the level of cytosolic transglutaminase activity 'in vitro' and 'in vivo'. Teale and Rees, 1987, reported on the immunogenicity and NK cell susceptibility of the hamster fibrosarcoma cell lines. They found that the highly metastatic lines were only weakly susceptible to NK cell cytotoxicity and were non-immunogenic, whilst the weakly metastatic lines proved to be immunogenic and NK sensitive. Another finding of interest was that tumours that were normally only weakly metastatic became highly metastatic when grown in immuno-surpressed hamsters, indicating that the cells were capable of undergoing all the stages of metastasis when protected from immune attack (Teale and Rees, 1987). From these findings, it can be suggested that the role played by the cytosolic form of transglutaminase in the mediation of metastatic ability is one of immune protection. If NK and LAK cells kill by initiating apoptosis, those cells whose apoptotic machinery is impaired should have a greater chance of survival, both in the tumour mass and in the circulation.

Further evidence to support the theory of an indirect relationship between transglutaminase activity and metastatic potential came from monitoring the level of an endogenous product of the action of transglutaminase, namely the protein crosslink $\epsilon(\gamma$-glutamyl)lysine. In those cell lines (parent, Met E and Met F) and tumours (parent, Met B and Met F) assayed, the amount of $\epsilon(\gamma$-glutamyl)lysine present was directly related to the level of activity of the cytosolic form of transglutaminase. If the production of $\epsilon(\gamma$-glutamyl)lysine is due solely to cytosolic transglutaminase activity, then calculation of a 'specific activity' for its production by the cytosolic enzyme (pmoles $\epsilon(\gamma$-glutamyl)lysine per unit activity) should give a figure that is consistent both between hamster fibrosarcoma cell variants and during

hamster fibrosarcoma growth. Both in the 'in vitro' and 'in vivo' situations, the production of $\epsilon(\gamma$-glutamyl)lysine by the cytosolic enzyme form appears to be fairly consistent between the variant cell lines (Table 8.3) and during tumour growth (Table 8.4). When specific activity is calculated per unit of total activity, the values obtained become more deviant suggesting that the particulate form of transglutaminase plays little or no part in the production of $\epsilon(\gamma$-glutamyl)lysine crosslinks. It is interesting to note the striking difference between the 'specific activity' of the enzyme 'in vivo' and 'in vitro', further demonstrating the difficulties inherent in predicting 'in vivo' behaviour from 'in vitro' results. Such differences could be caused by a low level of suitable substrate in the cultured environment, perhaps the lack of extracellular matrix, which would be present under normal conditions of growth. By comparing the amount of $\epsilon(\gamma$-glutamyl)lysine present in cultured cells and the apoptotic bodies isolated from cultured cells, it is apparent that the great majority (>80%) of the $\epsilon(\gamma$-glutamyl)lysine in cells is to be found within apoptotic bodies. Further, the high concentration of $\epsilon(\gamma$-glutamyl)lysine in the proteins of the apoptotic bodies indicate that these structures contain highly crosslinked proteins; in comparison, the concentration of $\epsilon(\gamma$-glutamyl)lysine in the proteins of cells not undergoing programmed cell death is low. There is therefore strong evidence to suggest that the major role of cytosolic transglutaminase is in the crosslinking of proteins to form the insoluble matrix of apoptotic bodies.

Table 8.3 : Specific activity of transglutaminase for the production of ϵ (γ-glutamyl)lysine, 'in vitro'.

Variant	pmoles ϵ(γ-glutamyl)lysine per unit activity	
	total activity	cytosolic activity
Parent	13.7 + 2.2	17.6 + 1.7
Met E	5.65 + 1.5	14.9 + 2.4
Met F	9.21 + 1.1	14.1 + 1.8

Specific activities were calculated from the measured activities given in Tables 3.3 and 3.4, and the levels of ϵ(γ-glutamyl)lysine given in Table 3.8.

Table 8.4 : Specific activity of transglutaminase for the production of ε (γ-glutamyl)lysine, 'in vivo'.

Variant	Tumour age / days.	pmoles ε(γ-glutamyl)lysine per unit activity: total activity	cytosolic activity
Parent	20	32.2 + 2.6	36.4 + 1.5
	24	31.9 + 1.4	36.9 + 2.7
	28	33.2 + 2.7	36.7 + 2.2
	34	33.4 + 2.1	37.9 + 2.8
Met B	24	23.2 + 2.8	32.8 + 4.9
	28	18.3 + 1.7	36.5 + 1.2
	34	14.5 + 1.3	36.8 + 2.4
Met F	24	23.8 + 2.7	33.8 + 3.3
	28	20.3 + 2.2	38.8 + 2.5
	34	15.2 + 1.8	37.2 + 2.6

Specific activities were calculated from the measured activities given in Tables 3.5 and 3.6, and the levels of ε(γ-glutamyl)lysine given in Fig.3.7.

Initial investigations into the expression of tissue transglutaminase in the cytosol, using antibodies raised against the cytosolic enzyme, revealed no variations in expression of cytosolic antigen between variants or during tumour growth. This indicated the occurrence of an inactivation of the cytosolic enzyme. Since measurements of activity were carried out on enzymes semi-purified by anion-exchange chromatography, inhibition of apparent enzyme activity, within the 'in vitro' assay, by endogenous polyamines seems unlikely. Similarly, the mimicking of alterations to cytosolic activity by alterations in the level of endogenous product also tend to refute the possibility of the measured activity losses being artefactual. By virtue of the cross-reactivity of the anti-cytosolic antibodies with particulate transglutaminase it was possible to ascertain the antigenic distribution of transglutaminase in the same way as the distribution of activity had been monitored. These investigations led to the discovery of a protein capable of cross-reacting with anti-cytosolic transglutaminase antibodies but showing no transglutaminase activity. The presence of this protein, found to be cytosolic in location, would explain why the expression of transglutaminase within the cytosol, as measured by polyclonal antibodies, does not vary. In fact, a direct relationship exists between the level of this inactive, yet antigenic, transglutaminase and the expression and activity of cytosolic transglutaminase, suggesting that the formation of the inactive protein may be the cause of the reduction in cytosolic activity.

Various workers have found an activation of transglutaminases after proteolytic action. For instance, Factor XIIIa is the active plasma transglutaminase produced by the cleavage of the inactive zymogen with thrombin (Takagi and Doolittle, 1974); Chung et al, 1988,

obtained an activated transglutaminase of 50kD by the treatment of the particulate form of tissue transglutaminase with a variety of proteases, although similar treatment of the cytosolic form led to its inactivation; Negi et al, 1985, produced an activated epidermal enzyme of 50kD by partially proteolysing a large inactive form. Treatment of cells with proteolytic agents has also been demonstrated to increase transglutaminase activity (Birckbichler et al, 1977; Korner and Bachrach, 1987). Proteolytic cleavage of the inactive antigenic protein led to its activation as a transglutaminase; with the resulting protein behaving in a manner similar to that of the cytosolic tissue transglutaminase under a number of biochemical and immunological asays, such as gel filtration and anion-exchange chromatography, denaturing and non-denaturing polyacrylamide gel electrophoresis and calcium activation.

Characterisation of the inactive antigen and its activated form indicated proteins of approximately 120kD and 80kD respectively, containing epitopes recognised by poly- and mono- clonal antibodies raised against cytosolic transglutaminase. Western blots of tryptic digests of the inactive antigen and the particulate and cytosolic forms of tissue transglutaminase revealed a high degree of homology between the cytosolic enzyme and the inactive protein, giving further evidence that these two proteins are closely related and also suggesting that they are the products of the same gene, possibly with the higher molecular weight inactive form being produced by inappropriate mRNA splicing events. For instance, the gene for acetyl-CoA carboxylase contains two promoter regions giving rise to at least five different forms of carboxylase mRNA by differential splicing of the two transcripts formed under the influence of the two promoters (Luo and

Kim, 1990). Such a mechanism could produce a protein with repeated epitopes and this would explain the observed increase in the reactivity of the inactive form with polyclonal anti-cytosolic transglutaminase antibodies compared to the cytosolic form. Indeed the tryptic digest map of the inactive protein indicates a peptide of approximately 30kD that reacts very strongly with polyclonal anti-cytosolic antibody and that is notable in its absence from the cytosolic map. Purification of this 30kD fragment followed by amino acid sequencing might help to elucidate the origin of the inactive transglutaminase. Since it is apparent that this form is an abnormallity associated with the highly malignant phenotype, understanding the mechanisms involved in its formation might lead to the discovery of a means of reversal leading to a reduction in metastatic ability.

Other potential causes of the formation of the inactive enzyme include the binding of extraneous material during homogenisation, the binding of a novel protein produced by abnormal cells, inappropriate post-translational modification and alterations to the tertiary structure of the protein caused by slight alterations in the amino acid sequence. Taking each in turn, the formation of the inactive protein is unlikely to be an artefact produced during homogenisation as its presence is undetectable in normal tissues and cells treated identically to malignant tissues and cells. Similarly, non-metastatic neoplastic tissues do not appear to contain the inactive protein and so the binding of a novel protein produced by transformed cells to the cytosolic form is unlikely to be the case. These mechanisms are also ruled out by the molecular weight determinations which indicate a Mr of 120 kD for the protein both by reducing SDS PAGE and gel filtration. If extraneous material was bound to the protein,

denaturation with SDS and DTT should remove it and therefore reduce the observed molecular weight. However, if transglutaminase were to act as its own substrate, proteinacious material could conceivably be bound to it thus inactivating it and increasing its molecular mass. Again, there seems no reason why this mechanism should not work in a normal or transformed cell. Production of an inactive transglutaminase by this mechanism would also make its activation to an apparently normal cytosolic form by simple proteolysis less likely since the isopeptide bond formed is resistent to general proteolysis. However, cleavage of the incorporated amine at a site close to the dipeptide link could be enough to restore activity. Inappropriate post-translational modifications, such as the addition of fatty acids, cannot yet be ruled out. Carbohydrate addition may also occur, although the sugars mannose, glucose and galactose do not appear to be present on any of the three forms of tissue transglutaminase. Slight alterations to the amino acid sequence of the cytosolic form leading to the formation of an inactive enzyme are feasible, however they are unlikely to alter the tertiary structure to such an extent (and in such a way as to be resistant to denaturing substances) as to produce an apparent increase in molecular mass of 40kD. Similarly proteolysis of such an altered protein would not produce an active enzyme of similar properties to the original enzyme. All of the potential mechanisms for the production of the inactive form mentioned in this paragraph would also be likely to alter epitope configurations and therefore reduce, or remove, the reactivity of the protein with the anti-cytosolic antibodies. Since this is not the case, none of these mechanisms provide an ideal hypothesis for the formation of the inactive protein.

Controversy still exists as to whether or not the particulate

form of tissue transglutaminase is a distinct enzyme, a post-translationally modified version of the cytosolic enzyme, or merely an artefact of homogenisation. Cross-reactivity of the particulate form with anti-cytosolic antibodies indicates that there are similarities between the two enzyme forms, although this could be due to a common ancestral gene and is not definitive proof that the two enzymes are one and the same. Differences in the calculated molecular weight of the enzyme, with gel filtration giving an Mr approximately 15kD higher than the estimate obtained from SDS PAGE, suggest that the particulate form does have material associated with it. The revelation of the epitope, on the particulate enzyme, for the monoclonal anti-cytosolic antibody by lipase or hydroxylamine further suggests lipid and fatty acid association. Harsfalvi et al, 1987, have reported on the addition of fatty acids to tissue transglutaminase and epidermal transglutaminase is known to have myristate and palmitate bound (Chakravarty and Rice, 1988), therefore the mechanism exists to modify the transglutaminase proteins with fatty acids. Similar effects produced by collagenase give credance to the suggestions that the particulate enzyme is merely the cytosolic form associating with collagen and other proteinacious material upon homogenisation. However, if the particulate enzyme is caused by the association of cytosolic transglutaminase with extracellular proteins, one might expect to see significant alterations in its apparent expression between individual tumours and tissues, whereas in fact, no significant differences are seen. Proponents of the theory that the particulate enzyme is an artefact, cite genetic studies in which only one message coding for tissue transglutaminase is found (section 1.4). These investigations, however, have been geared towards finding the message for the cytosolic form, rather than the

particulate; the initial cDNA probes were developed around known sequences, ie the active site, of the cytosolic form, which may not show suitable homology with the particulate sequence, and RNAase protection assays, when used, would, by definition of the assay, remove any mRNA that was not identical to the cDNA probe being used resulting in the detection of only one message. In a leukemic cell line, K592, 2 bands of mRNA were found to react with a cDNA probe for tissue transglutaminase, one band at approximately 4kB (the normal mRNA for the cytosolic form) and a larger band at approximately 7.4kB. Retinoic acid was seen to increase the level of expression of the 7.4kB mRNA and increase transglutaminase activity. Proliferation was also increased, but no differentiation was initiated nor was apoptosis apparent (Birckbichler, personal communication, 1990). Since apoptosis is connected with cytosolic transglutaminase activity, and an increase in the expression of soluble tissue transglutaminase accompanies the differentiation of monocytes (section 1.3.4) it would seem possible that the increase in transglutaminase activity seen in this case is due to the particulate form, rather than the cytosolic, and that this enzyme is coded for by a 7.4kB gene. Due to the size of this message, it would be tempting to suggest that it encodes the inactive transglutaminase protein. However, increases in its expression are accompanied by increases in activity, with no alterations to the expression of the smaller message, therefore the transglutaminase protein produced must be an active form. Thus the genetic evidence available at the moment is not definitive as to the existence of only one tissue transglutaminase. Further evidence to suggest that the particulate form is a separate enzyme comes from activation studies. Partial trypsinisation of the cytosolic enzyme leads to its almost

total inactivation whilst similar treatment of the particulate form produces a more active enzyme whose molecular weight has been estimated by Chung et al, 1988 to be 50kD. If the two forms were one and the same then partial proteolysis should have similar effects, unless extraneous material binds in such a way as to protect a trypsin susceptible site on the particulate enzyme.

From the results presented in this thesis, it is apparent that changes in tissue transglutaminase may play a role in the metastatic process and in the increasing malignancy of tumours during their growth. Further studies aimed at understanding the control mechanism of transglutaminase activation and inactivation might throw more light on the progression of neoplasms and on the cellular role or roles of the tissue transglutaminase or transglutaminases. It would be interesting to note if those control mechanisms involved in cytosolic transglutaminase activation/inactivation have similar effects on other processes involved in apoptosis, such as endonucleases. If a cells inability to form an apoptotic membrane is to confer any growth advantage or immunological protection then other apoptotic mechanisms might also be expected to be impaired. Information about the importance of cytosolic transglutaminase activity in metastasis, and its possible modulation by inactivation, could be obtained by transfection of a highly metastatic cell line with a suitable vector coding for the active cytosolic enzyme. Studies on activity and antigen distribution in transfected cells (or hybrids of a highly metastatic cell and a normal cell) might indicate the mechanism of formation of the inactive transglutaminase form. On the assumption that formation of the inactive protein is due to a malfunctioning of the cells original cytosolic transglutaminase gene, transfected and non-transfected cells could then

be described by the parameters of apoptotic rate, tumourigenic ability, invasive capacity, metastatic ability, and susceptability to LAK and NK attack. Comparison of these parameters might then provide information as to the importance of tissue transglutaminase in tumourigenesis and metastasis. On the clinical side, future studies should aim to further develop an assay either for the inactive protein, or for apoptosis, as an aid to diagnosing the metastasising ability of a tumour.

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Reduction in transglutaminase activity associated with tumour metastasis is due to the presence of an inactive form of the enzyme

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Post-translational modification of proteins is a vital physiological process stabilizing tissues and extracellular matrices. One group of enzymes involved in this process is the calcium-dependent acyl transferases known as the transglutaminases, which catalyse the formation of $\varepsilon(\gamma$-glutamyl) lysine bridges between proteins [1]. Several forms of transglutaminase have been identified, one of which is the tissue transglutaminase which is thought to be involved in a number of calcium-mediated cytoskeletal processes important to cell membrane functions [2–4]. This transglutaminase appears to exist in two forms: a soluble cytosolic enzyme and an insoluble membrane-associated enzyme [5].

It has been observed that, when compared with their normal equivalents, neoplastic tissues show decreased levels of transglutaminase activity [6, 7], and that further reductions in activity occurring within the primary tumour may be associated with the phenotypic alterations necessary for tumour progression and metastasis [8, 9]. More recent work

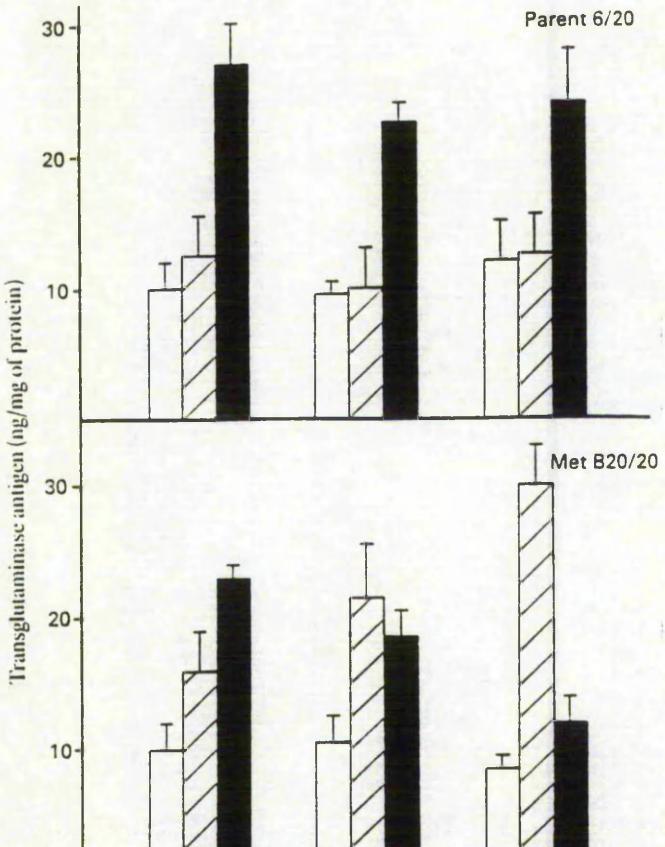

Fig. 1. *Levels of the three forms of transglutaminase antigen, found in a herpes simplex virus-2-induced hamster fibrosarcoma and its highly metastatic variant during tumour growth*

The metastatic potential of the two fibrosarcomas is indicated by the number of animals showing metastases out of 20 animals examined. Antigen levels are assessed by a quantitative sandwich e.l.s.a. Open bars represent particulate transglutaminase, hatched bars represent inactive antigen and filled bars represent cytosolic transglutaminase. Results are means ± S.E.M. ($n = 3$).

has indicated the existence of an indirect relationship between transglutaminase activity and the metastatic potential of a tumour [10] and that reductions in transglutaminase activity are due to decreases in the activity of the cytosolic form of the enzyme [9, 11].

To further this work, we have studied the distribution of antigenic transglutaminase within a poorly metastatic herpes simplex virus-2-induced fibrosarcoma, a highly metastatic variant of this fibrosarcoma and normal liver.

Tumours were grown in Syrian hamsters following subcutaneous injection of 10^4 cells. At 20, 28 and 34 days postinnoculation, non-necrotic tumour tissue was removed and homogenized by a Potter–Elevehjem in homogenizing buffer (0.25 M-sucrose, 2 mM-EDTA, 1 mM-dithiothreitol, 5 mM-Tris/HCl, pH 7.4, 5 mM-benzamidine, 1 mM-phenylmethanesulphonyl fluoride ~~PMSF~~), and fractionated into particulate and cytosolic fractions by centrifugation at 71 000 $g_{av.}$. The particulate fraction was then resuspended in homogenizing buffer plus 1% (v/v) Lubrol-PX, extracted on ice for 1 h and then fractionated as before into particulate and solubilized fractions. Both 71 000 $g_{av.}$ fractions were combined, dialys against 2 mM-dithiothreitol, 1 mM-EDTA, 5 mM-Tris/H pH 7.4, and filtered through 0.45 μm filters. Filtrates w then applied to a Mono-Q anion-exchange column a eluted with a 0–0.5 M-NaCl gradient. Fractions were c lected and assayed for transglutaminase activity as previou described [3] and transglutaminase antigen by a quantitat sandwich enzyme-linked immunosorbent assay (e.l.i. which employed an affinity-purified goat anti-guinea pig li transglutaminase antibody, a rabbit anti-rat liver tra glutaminase antibody and purified rat liver cytosolic tra glutaminase as a standard. Liver tissue was treated in identical fashion.

As previously observed [11], the activity profile for bo the tumours and liver, indicated two forms of tra glutaminase: the particulate form eluting at 0.15–0.25 NaCl and the cytosolic form eluting at 0.35–0.45 M-Na During growth of the poorly metastatic parent tumour, significant ($P < 0.05$) alteration to the levels of particul and cytosolic activity occurred. For the highly metasta variant Met B, particulate activity remained constant dur growth, while cytosolic activity decreased significan ($P < 0.05$).

The antigen profile for liver gave a similar pattern to t for activity, with two antigen peaks corresponding to activity peaks of the particulate and cytosolic forms. Ho ever, the antigen profiles for the tumours indicated tha third antigenic, but inactive, form of transglutaminase v present, eluting at 0.28–0.32 M-NaCl. The level of p ticulate antigen remained constant, both during tumo growth and between tumour lines. In the parent tumour li the levels of cytosolic and inactive antigen also remain unchanged during tumour growth. In the highly metasta variant Met B, the level of cytosolic transglutaminase antig decreased significantly ($P < 0.05$) with tumour progressio while the inactive form increased significantly ($P < 0.0$ (Fig. 1).

Our data, obtained from a poorly metastatic fibrosarco and its highly metastatic variant, suggest that the decrease cytosolic activity observed with tumour progression a increasing metastatic potential may be due to the formati of an inactive transglutaminase, either by post-translatio modification or by aberrant transcription of genom material.

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Received 20 November 1989

Volume 265, number 1,2, 93–96 FEBS 08463

Immunological similarities between cytosolic and particulate tiss transglutaminase

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Received 1 April 1990

At the present time it is uncertain whether or not the cytosolic and particulate forms of tissue transglutaminase are distinct and discre In this study a number of physical and immunological similarities between the two forms are demonstrated, indicating that they share sor epitopes, although their native confirmations may differ.

Cytosolic transglutaminase; Particulate transglutaminase

1. INTRODUCTION

Transglutaminases are a group of acyl-transferases that catalyse the post-translational cross-linking of proteins via the formation of $\epsilon(\gamma$-glutamyl)lysine bridges [1]. These enzymes exist in both extracellular forms (Factor XIII and prostate transglutaminase) and intracellular forms (epidermal, hair follicle and tissue transglutaminase) [2–4]. Tissue transglutaminase is thought to exist in two distinct forms, a predominantly soluble cytosolic enzyme and an insoluble particulate enzyme [5,6]. Although no role has as yet been confirmed for either form, it has been suggested that they may be involved in a number of calcium-mediated processes associated with the cell membrane and cytoskeleton [7–11], and with programmed cell death (apoptosis) [12]. Before the function of tissue transglutaminases can be fully understood, it is necessary to ascertain the relationship between the two forms since, at the present time, it is uncertain whether or not they are distinct and discrete enzymes. The aim of this study is to investigate any immunological similarities that may exist between the two enzyme forms.

2. EXPERIMENTAL

Cytosolic and particulate transglutaminase were semi-purified from rat liver homogenised to 20% (w/v) in buffered non-ionic detergent (1% Lubrol-PX in 0.25 M sucrose, 5 mM Tris, pH 7.4, 2 mM EDTA, 2 mM DTT, 1 mM PMSF) followed by anion exchange (Mono-Q column) chromatography [13]. Transglutaminase activity was measured by the Ca^{2+} dependent incorporation of [^{14}C]putrescine into *N,N*-dimethylcasein [13], and antigen was measured by a quantitative sandwich ELISA, utilisi purified goat anti-guinea pig liver cytosolic transglutamina bit anti-rat liver cytosolic transglutaminase [13]. Af against 5 mM Tris, pH 7.4, 2 mM EDTA, 2 mM DT purification of each enzyme form was carried out using a ty column of rabbit anti-cytosolic transglutaminase antib to cyanogen bromide activated Sepharose. Antigen was e 10% (v/v) dioxane in 0.25 M glycine/HCl, pH 2.5, then i neutralised with 2.5 M Tris, and desalted to remove the a Pharmacia PD-10 column using 5 mM Tris, pH 7.4, 2 as the eluting buffer. Purified fractions were then freeze- denatured samples of affinity purified particulate and cyto glutaminase were electrophoresed on 7.5% (w/v) acrylar non-reducing conditions and then Western blotted onto ni [14]. Samples, denatured by boiling in 40% (w/v) SDS, 2-mercaptoethanol, were electrophoresed on 10% (w/v) gels under reducing conditions [15] and then Western b nitrocellulose paper [14]. Western blots were immunoprob affinity purified polyclonal anti-cytosolic transglutamina raised in goat, or mouse monoclonal anti-guinea pig cyto glutaminase antibody, and visualised using a Biorad b tavidin HRP amplification system, as per the ma directions. Prior to immunoprobing with the monoclona some Western blots of non-reduced particulate enzyme v at 37°C for 1 h with 0.5 units in Tris-buffered saline (TB Tris-HCl, pH 7.4, 150 mM NaCl) plus 10 mM $CaCl_2$ lipase, collagenase, mannosidase, glucosidase or galactos control, Factor XIII (Boehringer), electrophoresed and W ted under comparable conditions, was also subjecte treatments. Western blots of cytosolic and particu glutaminase obtained from non-denaturing polyacrylamic also probed with the HRP labelled lectins Concanavalin *vulgaris* and *Bandeiraea simplicifolia*, in TBS plus 10 10 mM $MgCl_2$ and 10 mM $MnCl_2$.

3. RESULTS

As previously demonstrated [5,6], the cyto particulate transglutaminases are easily sepa anion exchange (Mono-Q) chromatography, particulate form eluting at 0.15–0.25 M NaC cytosolic form eluting at 0.35–0.45 M NaCl (

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

Similarly, the two forms also differ in their elution pattern from a rabbit anti-cytosolic transglutaminase affinity column, with the cytosolic form eluting first, after 2 column volumes of eluent (6 ml), and the particulate form eluting after 4 column volumes (12 ml).

Antigen profiles of anion exchange eluent fractions indicated that both affinity-purified goat anti-cytosolic transglutaminase and rabbit anti-cytosolic transglutaminase cross-reacted with the particulate form (Fig. 1B). However, if the monoclonal anti-cytosolic transglutaminase antibody was used as either the capture antibody or the visualising antibody in the sandwich ELISA assay, only the cytosolic peak was observed (Fig. 1C), indicating non-reactivity of the monoclonal antibody with the particulate form.

Western blots of non-denaturing polyacrylamide gels, immunoprobed with polyclonal affinity purified goat anti-cytosolic transglutaminase confirmed the expected differences in the relative electrophoretic mobilities of the two forms but further demonstrated the cross-reactivity of the antibody with both the particulate and cytosolic forms. Similar blots immunoprobed with monoclonal anti-cytosolic transglutaminase only demonstrated cross-reactivity with the cytosolic form (Fig. 2). However, when Western blots obtained from denaturing SDS-polyacrylamide gels were immunoprobed with the monoclonal antibody, both cytosolic and particulate forms were demonstrated (Fig. 3). Furthermore, the relative mobilities and calculated molecular masses (87 kDa in each case) were found to be comparable.

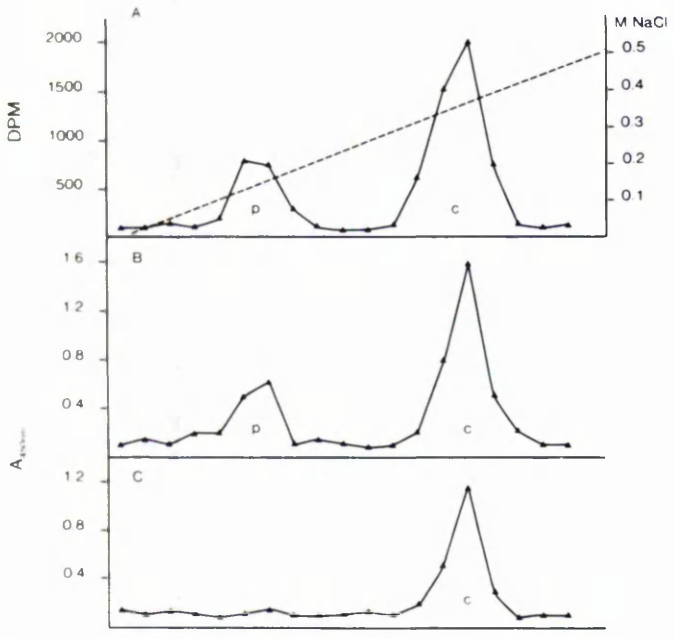

Fig. 1. Elution pattern of the particulate (p) and cytosolic (c) forms of transglutaminase on anion-exchange Mono-Q column (total elution volume 10 ml). (A) The enzyme activity profile. (B) Antigen profile using polyclonal antibody. (C) Antigen profile using monoclonal antibody.

Prior treatment of Western blots obtained from denaturing polyacrylamide gels with either lipas collagenase also led to cross-reactivity of the partic form with the monoclonal antibody (Fig. 2). The u denaturing gel electrophoresis or the enzyme treat of blots of the plasma transglutaminase Factor XII not produce a form capable of cross-reactivity either the monoclonal or polyclonal antibodies not shown). Mannosidase, galactosidase or glucosi treatment of Western blots did not produce a ticulate form capable of cross-reacting with monoclonal. Western blots of the particulat cytosolic transglutaminase previously separated non-denaturing polyacrylamide gel electrophoresis probed with the HRP-labeled lectins, Concanavali

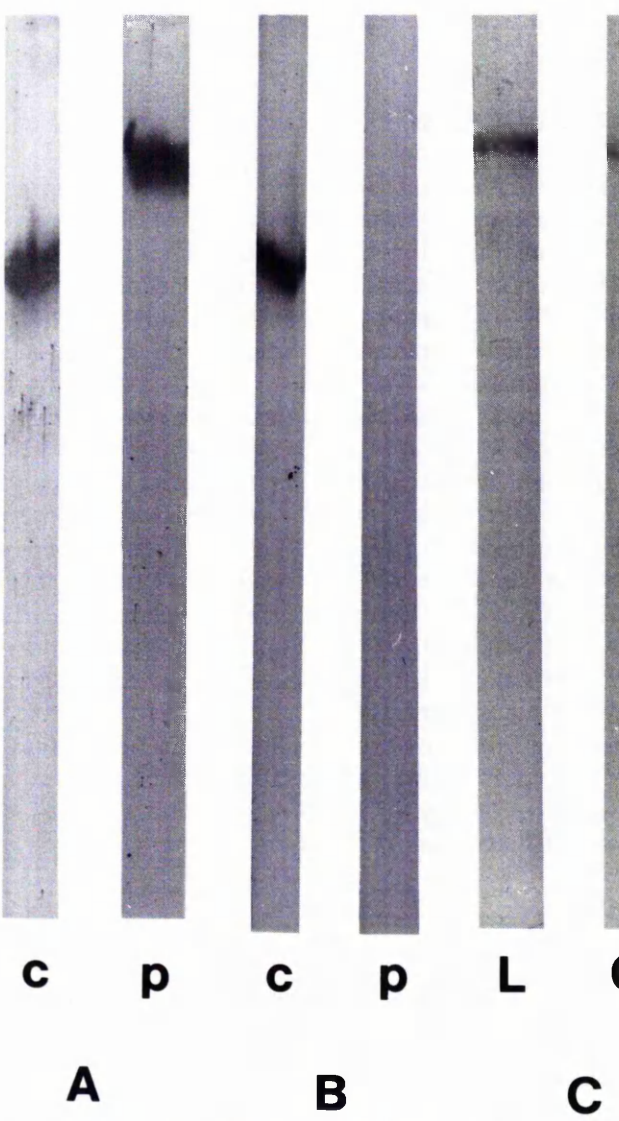

Fig. 2. Western blots of semi-purified (Mono-Q column) cytosc and particulate (p) transglutaminase following non-dena polyacrylamide gel electrophoresis. (A) Blots immunoprobed polyclonal antibody. (B) Blots immunoprobed with mono antibody. (C) Blots of the particulate immunoprobed monoclonal antibody following treatment with lipase (L) collagenase (Cg).

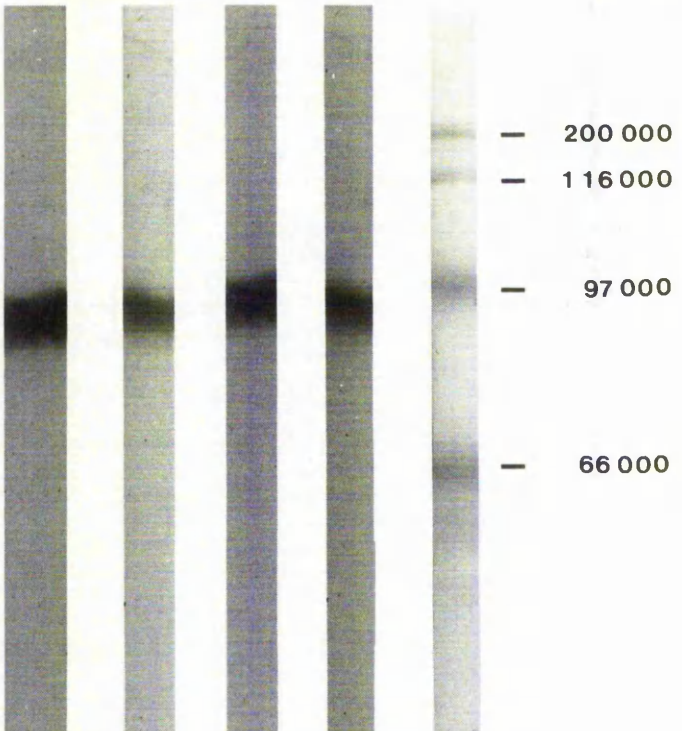

Fig. 3. Western blots of semi-purified (Mono-Q column) cytosolic (c) and particulate (p) transglutaminase following denaturing SDS-gel electrophoresis. (A) Blots immunoprobed with polyclonal antibody. (B) Blots immunoprobed with monoclonal antibody.

Triticum vulgaris and *Bandeiraea simplicifolia*, showed no reaction, confirming that mannose, glucose and galactose, respectively, were not components of either enzyme form.

4. DISCUSSION

Previous reports have indicated the molecular weight of the particulate transglutaminase, as calculated by size exclusion chromatography, to be 100 kDa, approximately 20 kDa greater than the known weight of the cytosolic form [5]. The data presented in this report indicate that when SDS-solubilised forms of the particulate and cytosolic transglutaminases are subjected to SDS-PAGE, their molecular masses appe comparable, at 87 kDa. On an immunological data also indicate that there is clearly a nu similarities between the two enzymes. The sug nose, glucose and galactose do not appear to b on either form, furthermore polyclonal antibo ed against the cytosolic enzyme also reacted particulate form, and with a greater avidity, gested by their relative elution patterns from cytosolic transglutaminase affinity column. with the monoclonal anti-cytosolic transglu antibody indicated that this antibody d cross-react with particulate transglutaminase its native form suggesting that the epitope missing from the protein or masked in so However, since SDS-denatured forms of the pa enzyme were found to react with this monocl tibody, both the cytosolic and particulate enzy possess the epitope for this antibody. Since d tion of the particulate enzyme reveals the epi suggestion is that in the native enzyme the e masked, either by further post-translational m tion as found in epidermal transglutaminase the binding of extraneous material during pu or by differences in the tertiary structure of th zymes. Since SDS PAGE indicated that the zymes have comparable molecular masses, contrasting with that obtained from chromatography (85 kDa for the cytosolic and for the particulate) each of the latter prop possible. Previous workers have indicated th transglutaminase has a high affinity for tracellular matrix [17–19] and suggestions h made that the binding of the cytosolic enzym lagen during cell fractionation may accou number of its particulate properties. The bi tissue transglutaminase to specific plasma m domains has also been noted [20]. The observa treatment with either lipase or collagenase cross-reactivity of non-denatured particula glutaminase with the monoclonal antibody that the particulate enzyme does indeed assoc both lipids and collagen, and that this associa be responsible for masking the epitope.

In conclusion, our data obtained fr munological studies indicate that the two tissue transglutaminase are far more closel than previously documented, each carrying a of similar epitopes.

Acknowledgements: We are grateful to Dr P.J.A. Davies a Birckbichler for the gifts of goat polyclonal anti-guine cytosolic transglutaminase antibody, and mouse monoc guinea pig liver cytosolic transglutaminase antibody, re and to the Cancer Research Campaign and the Yorksh Research Campaign who were in part responsible for the this work.

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Biochimica et Biophysica Acta, 1053 (1990) 13–20
Elsevier

BBAMCR 12689

The existence of an inactive form of transglutaminase within metastasising tumours

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(Received 5 October 1989)
(Revised manuscript received 18 January 1990)

Key words: Transglutaminase; Enzyme activity; (Hamster)

Separation by anion exchange chromatography of detergent extracts from a poorly metastatic HSV-2-induce fibrosarcoma, its highly metastatic variant and a highly metastatic rat fibrosarcoma indicated the presence of a form of transglutaminase antigen, when eluent fractions were assayed for transglutaminase activity and ant inactive antigenic transglutaminase was clearly separable from the particulate and cytosolic forms of glutaminase enzyme. Unlike tumours, its presence could not be demonstrated in extracts from normal Measurement of activity levels during tumour growth indicated that the progression of the two highly tumours was accompanied by a decrease in cytosolic transglutaminase activity, whilst the activity of this enz remained constant in the poorly metastatic tumour. Measurement of antigen levels indicated an inverse re between the level of inactive transglutaminase and the level of cytosolic transglutaminase activity, suggestin two forms are inter-related. Gel filtration indicated the molecular weight of the inactive form to be greater tha particulate and cytosolic forms, and it was estimated to be 120 000. Partial proteolysis of the semi-purifie form, by either trypsin or thrombin, led to its activation and to the appearance of a transglutaminase molecular weight and ionic mobility, both by anion-exchange chromatography and electrophoresis, to the transglutaminase.

Introduction

The calcium dependent transglutaminases catalyse an acyl-transfer reaction between the γ-carboxamide groups of protein-bound glutamine and primary amine groups. This results in the post translational modification of proteins either by the specific incorporation of amines or the crosslinking of proteins via ε-(γ-glutamyl)lysine bridges if the amine is the α-amino group of protein-bound lysine [1].

Various transglutaminases with distinct locations and structures have been identified, but only in plasma [2], epidermis [3] and semen [4] has a functional role been ascribed to their activity. The function of the tissue transglutaminase – the most widespread member of the group – remains poorly understood, although it has been implicated in the control of calcium-me membrane functions [5–14]. To further comp understanding of the cellular role of tiss glutaminase, recent evidence indicates the pr two active forms of the enzyme within cells, a and a membrane-bound form [15,16], thus necessary to qualify which form(s) is/are in any particular observations.

Previous investigations have suggested an a between reduced levels of enzyme activity, tu sis and tumour progression [17–20]. In hepa carcinomas this reduced activity appears to b specific lowering of the activity of the cytosol [16]. A further reduction in enzyme activity primary tumour may also be necessary for th nation of malignant cells from the prima during the metastatic process [17,21,22]. Us static variants of an HSV-2-induced fibrosar we have recently shown that decreased level glutaminase activity may be associated with metastatic potential [24]. In this study we have our investigations by ascertaining the level of

Abbreviations: HBBS, Hanks' balanced salts solution; PMSF, phenylmethylsulphonyl fluoride; DMSO, dimethylsulphoxide.

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of all antigenic forms of transglutaminase present in cells from a transplantable metastasising rat sarcoma and in a series of in vivo cloned HSV-2-induced hamster fibrosarcomas displaying varying metastatic potential [23].

Materials and Methods

Materials. [1,4-^{14}C]putrescine and [1,4(n)-^{3}H]putrescine were purchased from Amersham International, Little Chalfont, U.K.. *N,N'*-Dimethylcasein and all electrophoresis reagents were purchased from BDH Chemicals Ltd., Poole, U.K.. All other chemicals were purchased from Sigma Chemical Company Ltd., Poole, U.K. and were of the highest available grade.

Cell lines. The HSV-2-333-2-26 cell line (parent) was originally obtained by in vitro transformation of hamster embryo fibroblasts with inactivated HSV-2; this cell line was kindly provided by Dr. F. Rapp (Department of Microbiology Pennsylvania State University, Hershay, PA, U.S.A.). Sublines were derived from metastatic lung nodules in hamsters whose primary load had previously been resected; following in vivo passage, in vitro cultures were established [23].

Tumours. Cultured cells from two metastatic variants Met B (highly metastatic) and the Parent (weakly metastatic), were harvested, washed three times in Hanks' balanced salt solution (HBSS) and resuspended in HBSS at 10^5 cells per ml. Syrian hamsters were inoculated subcutaneously with 100 μl of viable cell suspension. Cell suspensions were assessed for viability by Trypan blue exclusion and only those preparations of more than 90% viability were used.

The propagation and origin of the transplantable rat sarcoma, P8 (a highly metastatic tumour) has been described elsewhere [25].

The developing tumours were removed at various time points, after the animals had been killed by dislocation of the neck. Non-necrotic tumour tissue was homogenised in a Potter-Elvehjem in buffer A (0.25 M sucrose, 2 mM EDTA, 1 mM dithiothreitol, 5 mM benzamidine, 1 mM PMSF, 1% (v/v) DMSO and 5 mM Tris (pH 7.4)) to 20% (w/v) and kept on ice.

Tissue fractionation. Tumour homogenates were fractionated into a cytosolic particles-free supernatant and particulate fraction by centrifugation at 71 000 × *g* for 45 min, in a Beckman ultracentrifuge.

Detergent extraction of tumour tissue. This extraction procedure is a modification of the method described by Chang and Chung [15]. The 71 000 × *g* particulate fraction was extracted three times with buffer B (buffer A containing 1% (w/v) Lubrol-PX). Each extraction was performed at 4° C for 45 min, following resuspension of the pellet in a volume of buffer equal to that of the original cytosolic particle-free supernatant. The buffer B extracts and the original cytosolic particle-free supernatants were then combined, dialysed against DTT, 1 mM EDTA, 5 mM Tris-HCl (pH 7.4 filtered through a 0.45 μm filter, ready for ani change chromatography.

Anion-exchange chromatography. The Phar FPLC system, with a Pharmacia Mono-Q colum utilised for separation of the two forms of glutaminase, as previously described [15]. Samp ml) were applied and eluted on a salt gradien 0–0.5 M NaCl in 2 mM EDTA, 5 mM Tris-HC 7.4) at a flow rate of 0.5 ml/min over 30 min. elution was monitored at 280 nm with a Pha UV-M monitor and collected as 1-ml fractions, were kept on ice.

Enzyme assays and chemical determinations transglutaminase activity present in tumour genates, subcellular fractions and pooled colum tions was measured by following the rate of inco tion of [^{14}C]putrescine into *N'N*-dimethylcasein, established methods [13]. Activity is expressed as 1 unit being equivalent to 1 nmol of putrescine in rated per hour under the conditions of the assa chromatography profiles, a modified assay wa ployed which utilised [^{3}H]putrescine at 2.05 Ci/ instead of [^{14}C]putrescine, for greater sensitivi other components were as before. Protein and were measured by the methods of Lowry et al. [2 Burton [27], respectively.

Quantitative ELISA for the detection of glutaminase antigen. A modification of the sar ELISA described by Fesus et al. [28] was use affinity-purified anti-guinea-pig liver transgluta raised in goat – courtesy of Dr. P.J.A. Davies, D ment of Pharmacology, University of Texas M School, Houston, TX, U.S.A. – was used as th mobilising antibody, and an anti-rat liver cy transglutaminase raised in rabbit (in our own l tory) was used as the detecting antibody. Anti- Horse-radish peroxidase conjugate was used to lise, with 3,3,5,5-tetramethyl benzidine as substra the peroxidase. Absorbances were read at 450 nm

Standard curves were set up using guinea-pi transglutaminase purified according to the meth Connelan et al. [29] and diluted in PBS prior to u

Controls were set up with PBS instead of th anti-guinea-pig liver transglutaminase, PBS as s PBS as rabbit anti-rat liver transglutaminase, goa immune serum instead of goat anti-guinea-pig transglutaminase and rabbit pre-immune serum i of rabbit anti-rat liver cytosolic transglutaminase

Molecular weight analysis. Molecular weights assessed by gel filtration on an LKB TSK SW column (7.5 × 600 mm) with a molecular weigh aration range of 5000–300 000. Elution was e isocratically with an M-45 pump (Waters) using sodium sulphate containing 5% (w/v) glycerol,

EDTA and 50 mM Hepes (pH 7.4). Samples (100 μl) were loaded and eluted at 0.3 ml/min. Protein was monitored at 280 nm using a Pharmacia UV-M monitor and 200 μl fractions were collected, which were kept on ice. The column was calibrated with proteins of known molecular weight: aldolase, bovine serum albumin (dimer), bovine serum albumin (monomer), ovalbumin, trypsinogen and β-lactoglobulin.

Polyacrylamide gel electrophoresis and western blotting. Electrophoresis of proteins was carried out under non-denaturing conditions using a 7.5% (w/v) acrylamide gel (7.5% (w/v) acrylamide, 0.1% (w/v) bisacrylamide, 0.4 M Tris-HCl (pH 8.9) in a Bio-Rad mini-gel system. Samples (10 μl) were diluted 1:1 in 10% (v/v) glycerol, loaded onto the gels and subjected to electrophoresis for 1 h at 175 V constant voltage. Gels were then either stained with Coomassie blue (0.1% (w/v) in aqueous 18% (v/v) methanol, aqueous 5% (v/v) acetic acid) or electroblotted onto nitrocellulose using an LKB dry-blot system and a 48 mM Tris/39 mM glycine buffer (pH 9). Western blots were immunostained with affinity-purified anti-guinea-pig liver cytosolic transglutaminase (raised in goat) which was then visualised using a Bio-Rad anti-goat Biotin/Streptavidin horse-radish peroxidase system, following the manufacturers instructions.

Proteolysis of transglutaminase. Limited proteolysis of the transglutaminase antigen, obtained by anion-exchange (Mono-Q) chromatography of detergent extracts from rat P8 fibrosarcoma (32 days) and concentrated by reverse dialysis using Sephadex G-25 (pharmacia), was carried out at 37°C for 10 min using 10 ng trypsin or 0.5 units thrombin/μg of transglutaminase. Prior to assaying trypsinised samples for transglutaminase activity, 1 ng trypsin inhibitor was added/ng of trypsin.

Statistical analysis. A two-sample *t*-test was used to compare the means. This analysis was carried out using the Minitab system (Minitab Inc., U.S.A.). W 0.05, the difference between the two means wa ered to be statistically significant. The Product-moment coefficient (r) was used as a tion coefficient: $r = x \cdot y/N \cdot S_x \cdot S_y$.

Results

Transglutaminase activity and antigen in an H duced hamster fibrosarcoma and its highly m variant

Initial studies were undertaken in order to activity and antigen levels for the different transglutaminase present in tumours.

Total transglutaminase protein was extract tumours, at 20, 28 and 34 days growth, using containing 1% (w/v) lubrol, and the extracts v separated by anion-exchange chromatography.

Fractions collected after anion-exchange c graphy were assayed for transglutaminase acti antigen. Cytosolic transglutaminase activity (el tween 0.35–0.45 M NaCl) in the highly metast B tumour was significantly lower ($P < 0.05$) t found in the weakly metastatic parent tum continued to decrease significantly during tum gression, whilst cytosolic activity in the paren remained constant during growth (Table I). Th particulate transglutaminase activity (eluting 0.15–0.2 M NaCl) did not vary significant between tumour lines or during tumour growt I). Measurement of transglutaminase antigen i tumours revealed three distinct peaks of an (Figs. 1 and 2). Two of these peaks correspond activity of the particulate and cytosolic form enzyme, respectively. This result indicated that clonal antibodies raised against the cytosolic were showing considerable cross-reactivity with

TABLE I

Levels of transglutaminase antigen and activity in hamster fibrosarcomas Met B and parent, and rat fibrosarcoma P8 at different stages of tu

Transglutaminase activity and antigen levels were measured in pooled fractions obtained from anion-exchange (Mono-Q) chromato shown in Figs. 1 and 2, according to the procedures described in Materials and Methods section. The cytosolic activity/antigen repr eluting between 0.35–0.45 M NaCl, the inactive antigen represents that eluting between 0.28–0.33 M NaCl and the particulate activ represents that eluting between 0.15–0.25 M NaCl plus that remaining in the extracted pellet. Data represent mean values of three experim except * where data represent the mean of six experiments ± S.E.

	Parent			Met B			P8	
	20 d [a]	28 d	34 d	20 d	28 d	34 d	15 d	32
Activity levels (U/mg DNA)								
particulate	157 ± 67	142 ± 103	124 ± 83	*198 ± 49	*284 ± 33	*254 ± 30	91 ± 22	80
cytosolic	1250 ± 124	1045 ± 114	941 ± 34	*600 ± 98	*309 ± 95	*167 ± 28	200 ± 15	60
Antigen levels (ng/μg DNA)								
particulate	2.4 ± 0.2	2.3 ± 0.2	2.0 ± 0.3	2.4 ± 0.2	2.6 ± 0.2	2.8 ± 0.2	1.1 ± 0.1	1.
inactive	2.5 ± 0.3	2.4 ± 0.2	2.7 ± 0.3	3.6 ± 0.4	5.8 ± 0.3	6.8 ± 0.2	3.0 ± 0.2	4.
cytosolic	6.1 ± 0.2	5.6 ± 0.4	5.9 ± 0.4	5.7 ± 0.1	3.6 ± 0.1	2.5 ± 0.2	2.1 ± 0.2	1.

[a] Age in days (d).

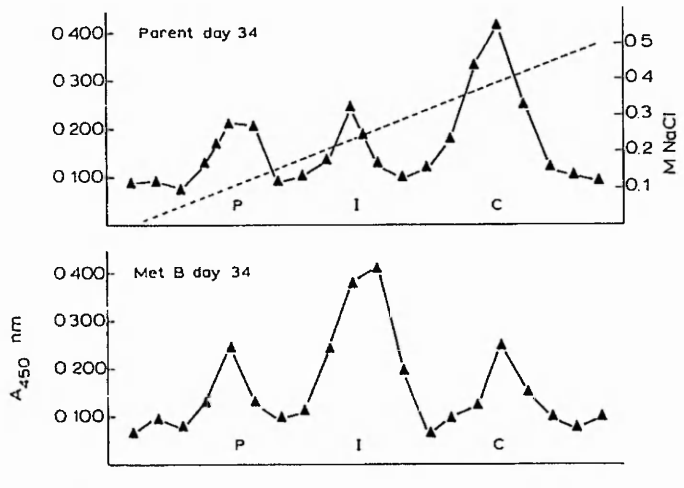

Fig. 1. Separation of transglutaminase antigen, present in HSV-2-induced hamster fibrosarcomas, by anion-exchange (Mono-Q) chromatography. Tumours were extracted with Buffer A containing 1% Lubrol PX as described in Materials and Methods. The elution profiles are representative separations of extracts from Met B tumour at 20 and 34 days growth, and from the parent tumour at 20 days growth. P and C refer to the elution positions of the particulate and cytosolic forms of transglutaminase respectively; I, refers to the inactive antigen. Antigen levels were measured using the sandwich ELISA assay described in Materials and Methods.

ticulate enzyme. The third antigenic peak, eluting at 0.3 M NaCl, showed no transglutaminase activity when assayed by the Ca^{2+}-mediated incorporation of ^{14}C putrescine into N,N'-dimethylcasein. Similar investigations undertaken on extracts from rat liver indicated that this inactive form of transglutaminase antigen was not present in this normal tissue (Fig. 2).

If it is assumed that the polyclonal antibodies (raised against the cytosolic enzyme from the livers of guinea-pig and rat) used in the detection of the different antigenic forms of transglutaminase, show comparable affinity for these different antigenic proteins, then an approximation of the relative amounts of each antigenic form can be ascertained.

Reference to Table I indicates that the expression of the particulate enzyme did not vary significantly either between tumour lines or during tumour growth. In the weakly metastatic parent tumour the level of expression of both the cytosolic enzyme and the inactive antigen remained stable during growth. In contrast, the growth and progression of the highly metastatic Met B tumour was accompanied by a significant ($P < 0.05$) increase in the level of inactive transglutaminase antigen. This increase was found to correlate ($r = -0.99$) with a significant ($P < 0.05$) decrease in the level of cytosolic glutaminase antigen.

Transglutaminase antigen in the rat fibrosarcoma

To test whether this inactive form of the e protein found in the hamster fibrosarcoma was in other malignant tumours, in particular those o in other animal species, its presence was investig the highly malignant rat fibrosarcoma P8, on d and 32 post tumour transplantation.

As found with the hamster fibrosarcomas, thre genic peaks were detected, after anion-exchang matography, (Fig. 2). Two of these peaks, elu 0.15–0.2 M NaCl and 0.35–0.45 M NaCl corresp the activity peaks of the particulate and cytosoli respectively, while the third antigenic peak sponded to the inactive form of the enzyme.

Quantitation of the antigen found in these p the two different stages of growth (Table I) indi significant ($P < 0.05$) reduction in the cytosolic and a corresponding increase in the inactive a while the levels of the particulate antigen did n significantly. As with the hamster fibrosarcoma

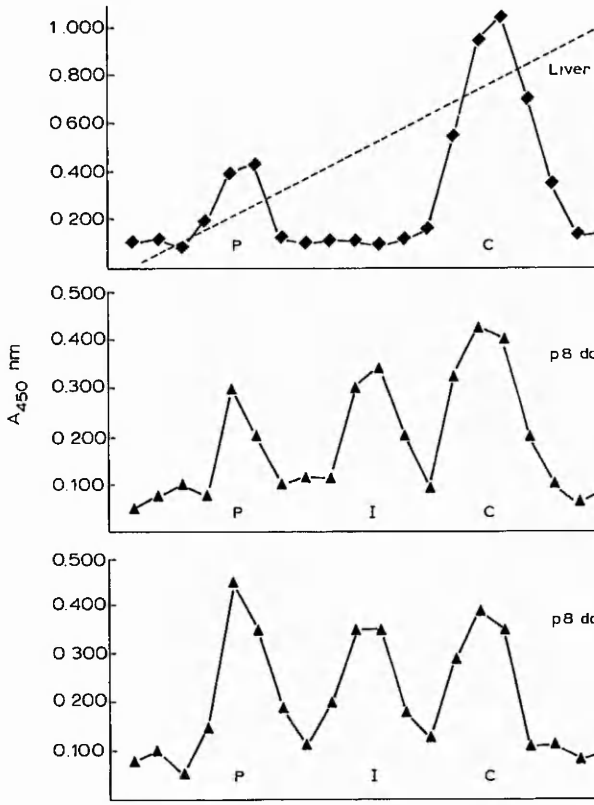

Fig. 2. Separation of transglutaminase antigen, present i fibrosarcoma and normal liver, by anion-exchange (Mono- matography. Tissues were extracted and antigen levels were as described in Fig. 1. The elution profiles are representa arations of tumour extracts obtained at 15 and 32 days gro normal liver. P and C refer to the elution positions of the p and cytosolic forms of transglutaminase respectively, I refe inactive antigen.

this changing profile of the cytosolic antigen during tumour growth corresponded to a reduction in the activity of the cytosolic enzyme.

Characterisation of the inactive form of transglutaminase antigen

Further characterisation of the inactive form of transglutaminase antigen was undertaken on tumour extracts obtained from the rat fibrosarcoma P8, harvested at 32 days growth.

To elucidate the subcellular localisation of the inactive form (whether cytosolic or particulate) tumours were homogenised in Buffer A and the homogenate separated into cytosolic and particulate fractions by centrifugation at $71\,000 \times g$ for 45 min. The resulting cytosolic fraction was then subjected to anion-exchange chromatography and the eluent assayed for transglutaminase antigen. The particulate fraction was then extracted with Buffer A containing 1% (w/v) lubrol and the $71\,000 \times g$ supernatant similarly treated. From these investigations it was apparent that the inactive antigen was predominantly (70%) cytosolic in localisation (data not shown).

The molecular weights of the cytosolic and particulate transglutaminases have been previously determined as 75 000–80 000 and 95 000–10 000, respectively [15]. To assess and compare the molecular weight of the inactive antigen, tumour extracts were separated by anion-exchange chromatography and the relevant fractions were pooled to give partially purified particulate, inactive and cytosolic protein fractions. The pooled fractions were then applied to a precalibrated TSK SW 3000 molecular seive column for measurement of their different molecular weights. Eluent fractions were assayed for transglutaminase antigen and the elution profiles gave molecular weights of 80 000 for the cytosolic enzyme, 100 000 for the particulate and 120 000 for the inactive transglutaminase antigen (Fig. 3).

The finding of an inactive enzyme protein, in tumour extracts, posessing a molecular weight greater than that of the cytosolic enzyme, and being cytosolic in localisation, suggested that this protein may be a precursor of the cytosolic form of the enzyme. Such a hypothesis would agree with the data shown in Table I, where growth of both the Met B and P8 tumours is accompanied by a decrease in the expression and activity of the cytosolic form. This, in turn, is accompanied by a corresponding increase in the level of the inactive enzyme protein.

Limited proteolysis using trypsin or thrombin was therefore undertaken on the semi-purified inactive antigenic form obtained from extracts of the P8 sarcoma (day 32). Measurement of [^{14}C]putrescine incorporation into N,N'-dimethylcasein by proteolysed antigen indicated that both these proteolytic enzymes were capable of restoring enzyme activity.

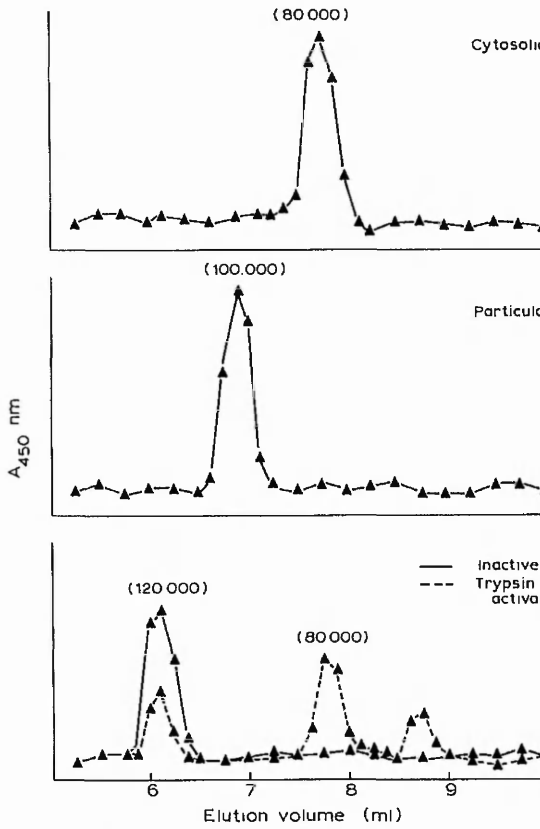

Fig. 3. Elution profiles for the different transglutamina present in rat P8 fibrosarcoma (32 days), and for the inac after trypsin treatment, following separation on a TSK molecular seive column. Pooled fractions of the diffe glutaminase antigens, obtained from anionexchange chro (Fig. 2), and inactive antigen treated with trypsin accor procedures described in the methods, were applied to a p TSK SW 3000 molecular seive column and eluted as desc methods section. Antigen levels were measured in the dif tions using the sandwich ELISA assay described in the M Methods. The broken line represents the elution profile of antigen after trypsin treatment. The calculated molecular shown in parenthesis.

Further characterisation of the proteolysed transglutaminase antigen was undertaken by ferent methods. The first involved separatio proteolysed mixture by anion-exchange chro phy. This gave rise to the presence of two peaks, one eluting at 0.25–0.3 M NaCl, corre to the inactive enzyme protein, and one e 0.35–0.45 M NaCl, corresponding to the cyt zyme (data not shown). Similarly, when the transglutaminase antigen was applied to a prec gel-filtration column, three antigenic peaks we table. One at 120 000, corresponding to the antigen, a major peak at 80 000, correspondi cytosolic enzyme, and a minor peak at 65 000 Further confirmation that partial proteolysis active antigen led to the appearance of ar protein corresponding to the cytosolic form tained by Western blotting non-denaturi

Fig. 4. Western blot of purified guinea-pig-liver enzyme (PC); particulate transglutaminase (P); inactive transglutaminase antigen (I) and trypsin treated inactive antigen (TI), immunoprobed with polyclonal anti-guinea pig liver cytosolic transglutaminase. The inactive antigen and the particulate transglutamine were obtained from the P8 fibrosarcoma (day 32) using anion-exchange chromatography (Fig. 2). Trypsin treatment of the inactive antigen and the electrophoretic separation of the different antigens were according to the procedures described in the Materials and Methods.

acrylamide gels of proteolysed inactive antigen (Fig. 4). Comparison of the lane corresponding to the trypsinised inactive antigen indicated a profile which was identical to that obtained from the purified guinea pig liver cytosolic enzyme. Interestingly, a small band was present in the particulate enzyme that corresponded to the mobility of the inactive enzyme form.

Discussion

In previous studies using metastasising sarcomas, we have suggested that the propensity of a tumour to metastasise may bear an inverse relationship to the levels of transglutaminase activity that it contains [24]. Similar results have also been demonstrated in other laboratories, using rabbit rhabdomyosarcomas [21]. In this study, we have observed that this relationship between metastatic potential and transglutaminase activity is due to changes in the levels of activity of the cy form of the tissue transglutaminase enzyme. Inv tions were therefore undertaken to establish a ca the reduced cytosolic transglutaminase activity ob in these tumours. Initial studies were undertal demonstrate the possible presence of an inactiv of transglutaminase antigen that was absent in n tissue. While undertaking these investigations, a n of interesting observations were made. Studie normal rat liver tissue indicated that this tissu tained two antigenic forms of transglutaminase, sponding to the cytosolic and the particulate fo the enzyme. Since the antibodies used to detect glutaminase antigen were raised against purifie solic enzyme from guinea-pig and rat liver, the de of antigenic particulate enzyme suggests that the enzyme forms are not immunologically distinct as ously indicated, but instead must share some co epitopes. We can only explain this discrepancy data to that previously published by the fact th ELISA method used in this study is far more se that the Ouchterlony diffusion method used in studies.

Of importance to the present study was the ob tion that, in normal liver, no inactive form of glutaminase antigen was detectable. This finding contrast to our investigations undertaken with e from malignant rat and hamster fibrosarcomas. E these tumours contained an inactive antigenic fo transglutaminase which was separable from the particulate and cytosolic enzymes by anion-exc chromatography. Furthermore, the level of inacti tigen appeared to be inversly related to both the l cytosolic antigen and activity that the tumou tained. This observation was clearly demonstrate ing the growth of the highly metastatic hamster sarcoma, Met B, and in tumour extracts obtaine the rat fibrosarcoma, P8, at early and late sta growth, with the level of the inactive form increa the expense of the active cytosolic form.

In contrast, in the less metastatic parent h fibrosarcoma, which shows no significant alterat cytosolic transglutaminase activity during growt level of inactive antigenic transglutaminase wa compared to that of the highly metastatic Met B tu and did not vary during tumour progression.

In all tumours examined, the levels of part transglutaminase antigen and activity did not va nificantly during tumour growth, further strengt the correlation between the cytosolic form an inactive form.

The finding of this inactive enzyme protein in nant tumours obtained from both rat and ha suggests that its presence may be common to a n of malignant neoplasms, where reduced tran minase activity has been observed [17–22].

Our data therefore suggest that the presence of the inactive enzyme protein in the metastatic tumour cell is likely to be a result of inappropriate expression of the cytosolic transglutaminase. Since the inactive enzyme protein is larger than either the particulate or the cytosolic enzyme, it is unlikely to be a proteolytic product of either form. Furthermore its inability to cross-react with antibodies raised against the a-subunit of Factor XIII indicated that it was immunologically distinct to this inactive zymogen (data not shown).

Further evidence to indicate that the inactive form of enzyme protein may be the result of inappropriate expression of the cytosolic enzyme came from studies involving its limited proteolysis. Treatment of the inactive protein with either trypsin or thrombin, led to the appearance of an active form of transglutaminase enzyme which displayed a number of biophysical properties comparable to that of the cytosolic enzyme. Using both anion-exchange and molecular seive chromatography, it was found that the major antigenic product resulting from partial proteolysis co-eluted with the cytosolic transglutaminase antigen. No antigen resembling that of the particulate enzyme was ever detectable in any of these investigations. Similarly, investigations using Western blotting, indicated that the electrophoretic pattern for the trypsinised inactive antigen was identical to that of the purified guinea-pig-liver cytosolic enzyme when proteins were separated by non-denatureing polyacrylamide electrophoresis. Interestingly, in the particulate enzyme a slower moving antigen band corresponding electrophoretically to that of the inactive enzyme was also detectable. However, the absence of the inactive antigen in particulate fractions collected from the anion-exchange column and further analysed by molecular seive chromatography suggest that this band is unlikely to be the inactive enzyme.

Our data therefore strongly suggest that, in the metastatic tumour cell, reduction in the level of the cytosolic enzyme protein is a consequence of its perturbed expression. The result is the production of an inactive enzyme protein with a molecular weight of approx. 40 000 greater than that of the cytosolic enzyme. The finding that limited proteolysis of this inactive protein leads to the formation of an active enzyme resembling that of the cytosolic transglutaminase, suggests initially that its accumulation in the metastatic tumour cell may be the result of inappropriate post-translational processing. However, sequence studies undertaken on the isolated cDNA for the guinea-pig-liver enzyme suggest that this is unlikely to be the case [30]. Primary extension studies undertaken on the 5′ end of the mRNA indicate the presence of only 110 nucleotide residues upstream from the translational start signal. Maximal translation of this mRNA transcript could therefore not lead to a pro-enzyme of approx. 40 000 greater in molecular weight than the active cytosolic enzyme. Furthermore, Northern blots undertaken with mR tracts from guinea-pig-liver have indicated the of only one stable form of mRNA correspond size of 3.7–3.8 kb [30]. In view of these finding be suggested that the large inactive enzyme found in the metastatic tumour cell is likely result of inappropriate gene expression whic either at the transcriptional or post-transcriptio possibly during mRNA splicing. The suggesti our data that a further mRNA transcript, codin inactive enzyme protein, is present in the m tumour cell is also intriguing, when it is consid the interrelationship between the particulate a solic forms of transglutaminase at the genetic still unknown.

Acknowledgments

We are grateful to the Cancer Research C and the Yorkshire Cancer Research Campa were in part responsible for the support of this

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MO 169

REDUCED TRANSGLUTAMINASE ACTIVITY MAY BE IMPORTANT
TUMOUR METASTASIS

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An important function of the tissue transglutam
may be concerned with the stabilisation of memb
or membrane associated proteins, through
crosslinking via ε(γ-glutamyl) lysine bridges. Ea
observations have indicated that neoplastic ti
show reduced transglutaminase activity when com
with their normal counterparts. Studies undertake
HSV-2 induced hamster fibrosarcomas of diff
metastatic potential have demonstrated that re
transglutaminase activity may also be important
tumour progression and metastasis. Levels
transglutaminase activity found in cell cultures o
parent tumour and two metastatic variants, Met B and
Met D, indicated an inverse relationship be
transglutaminase activity and metastatic poten
Measurement of transglutaminase activity in
tumours derived from cells of the parent tumour an
highly metastatic variants, Met B and Met F, conf
this observation in the 'in vivo' situation. Leve
the protein crosslink ε(γ-glutamyl) lysine were fou
be significantly reduced in tumours from the h
metastatic variants Met B and Met F when compared wi
the parent tumour, thus confirming the relatio
between measured enzyme activity and the endog
product of the enzyme. The propensity of a tumou
metastasise may therefore be related to the leve
transglutaminase that it contains.

Transglutaminase activity, tumour growth and metastasis

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Transglutaminases are calcium-dependent acyltransferases involved in the post-translational modification of proteins, either through the incorporation of primary amines (such as polyamines) into peptide-bound glutamine residues, or through the cross-linking of proteins via ε-(γ-glutamyl)-lysine bridges when the amine is peptide-bound lysine (Folk & Finlayson, 1977).

The most widespread member of this group, the tissue transglutaminase, is also the least understood: however, there is now accumulating evidence to suggest its importance may lie in the mediation of a number of calcium-regulated cytoskeletal processes important to cell membrane functions (Davies *et al.*, 1980; Loewy & Matacic, 1981; Bungay *et al.*, 1986; Owen *et al.*, 1988).

Of importance to the following work is the observation that neoplastic tissues show reduced transglutaminase activity when compared with their normal co (Birckbichler *et al.*, 1977; Barnes *et al.*, 1984). M studies have also indicated that a further reductio glutaminase activity in the primary tumour may be for the phenotypic characteristics associated wi progression and metastases (Barnes *et al.*, 1985; *al.*, 1986; Hand *et al.*, 1987).

In order to confirm this important observatio measured transglutaminase activity in a number o derived from the metastases of a herpes simpl induced hamster fibrosarcoma. Each cell line, alth nating from the same parent tumour, shows a distinct characteristics, one of which is differing potential (Teale & Rees, 1987).

Transglutaminase activity was measured bot derived from cell culture, and in tissue derivec growing tumour after subcutaneous injection of into Syrian hamsters. Cells were cultured in minim tial medium supplemented with amino acids, vit foetal calf serum. Confluent cells were harvested b washed three times in homogenizing buffer (0.25 2 mM-EDTA, 1 mM-dithiothreitol, 5 mM-Tris/H containing 5 mM-benzamidine and 1 mM-pheny sulphonyl fluoride) and then broken by sonication

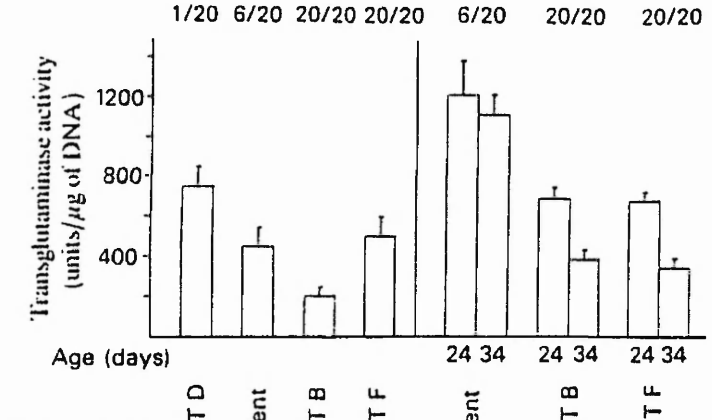

Fig. 1. *Levels of transglutaminase activity in metastatic variants of herpes simplex virus-2 induced hamster fibrosarcoma, after growth in cell culture, and in the host animal after subcutaneous injection*

The metastatic potential of each variant is indicated, above the histogram, by the number of animals showing metastases out of 20 animals examined. One unit of activity is equivalent to one nmol of putrescine incorporated/h. Results are means ± S.E.M. ($n = 3$).

6 μ in the same buffer. The resulting homogenate was assayed for transglutaminase activity as previously described (Bungay *et al.*, 1986). Tumours were harvested at 24 and 34 days after inoculation and the non-necrotic tumour tissue was homogenized by a Potter–Elvehjem in the same buffer used for cell cultures. Transglutaminase activity was then measured in the resulting homogenate.

Levels of transglutaminase activity found in the cell cultures (Fig. 1) indicated that for the parent tumour, and the two metastatic variants MET B and MET D, an indirect relationship existed between metastatic potential and levels of transglutaminase activity. However, this relationship did not hold for the highly metastatic variant MET F which showed comparable transglutaminase activity with the parent tumour. Since both transglutaminase activity and the propensity of a tumour to metastasize may be influenced by the external pressures exerted by the host animal, transglutaminase activity was also measured in tum from cells of the original parent tumour, and derived from the highly metastatic variants MET F. Interestingly, levels of transglutamin found in the parent tumour and in the tumour the metastatic variant MET B were found to be ($P<0.05$) higher than the levels found in cell observation we are unable to explain at the However, when grown in an environment influe host animal, the highly metastatic MET F tumo comparable transglutaminase activity to that metastatic MET B variant, with both tumour significantly ($P<0.05$) lower activity than the le parent tumour. Furthermore, during tumour gro significant reduction ($P<0.05$) in transglutami occurred in the two highly metastatic variants b less metastatic parent.

Our data, using metastatic variants of a ha sarcoma growing both in cell culture and in the therefore confirm and extend earlier observatio that the propensity of a tumour to metastasiz directly related to the levels of transglutaminase it contains.

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Received 28 November 1988

IS CYTOSOLIC TISSUE TRANSGLUTAMINASE ACTIVITY INV IN THE FORMATION OF APOPTOTIC BODIES IN PROGRAMME CELL DEATH?

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The activities of the two forms of tissue transglutaminase were measured in normal hamster fibroblasts and a series of metastatic variants o HSV-2 induced hamster fibrosarcoma. Cytosolic transglutaminase activity was seen to decrease wi increasing metastatic potential, and during tumou progression. A direct relationship between measur cytosolic activity and the levels of the endogeno product of transglutaminase, ε (γ glutamyl)lysine also observed. Initial studies also suggest that may be a correlation between the level of cytosol transglutaminase activity and the number of cells undergoing programmed cell death via apoptosis.