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Tissue transglutaminase in normal and abnormal wound healing: Review article

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Summary. A complex series of events involving inflammation, cell migration and proliferation, ECM stabilisation and remodelling, neovascularisation and apoptosis are crucial to the tissue response to injury. Wound healing involves the dynamic interactions of multiple cells types with components of the extracellular matrix (ECM) and growth factors. Impaired wound healing as a consequence of aging, injury or disease may lead to serious disabilities and poor quality of life. Abnormal wound healing may also lead to inflammatory and fibrotic conditions (such as renal and pulmonary fibrosis). Therefore identification of the molecular events underlying wound repair is essential to develop new effective treatments in support to patients and the wound care sector.

Recent advances in the understating of the physiological functions of tissue transglutaminase a multi functional protein cross-linking enzyme which stabilises tissues have demonstrated that its biological activities interrelate with wound healing phases at multiple levels. This review describes our view of the function of tissue transglutaminase in wound repair under normal and pathological situations and highlights its potential as a strategic therapeutic target in the development of new treatments to improve wound healing and prevent scarring.

Keywords: Tissue transglutaminase – Wound healing – Fibrosis – Fibronectin – Collagen

Overview of wound healing

Wound healing is characterised by three interrelated and overlapping phases: inflammation, tissue formation/stabilisation and tissue remodelling. It is a complex and dynamic process involving the interactions of different cell types that vary depending on the type of tissue involved. It employs an intercalating array of signalling peptides such as growth factors and cytokines that control and regulate a co-ordinated cellular and extracellular matrix remodelling to repair the lesion. For example, following injury to the skin, which is probably the most well characterised process (Singer and Clark, 1999; Harding et al., 2002 [and references therein]), the wound is normally filled by a blood clot which re-establishes hemostasis and serves as a temporary matrix for the migration of cells from adjacent tissue. It consists mainly of transglutaminase-mediated (factor XIIIa) cross-linked fibrin with incorporated plasma fibronectin (FN). In particular FN acts as a provisional matrix for cell migration and cell adhesion by interaction of its epitopes with transmembrane receptors. These are predominantly the integrin class of receptors, which mainly recognise the Arg GlyAsp (RGD) epitope within the FN type III_{10} domain and the heparan sulfate proteoglycans (HSPG) class of receptors (primarily syndecan-4), which mainly bind the FN C-terminal HepII epitope (Burridge et al., 2001). Cells within the thrombus, predominantly activated platelets, release many wound healing mediators (such as platelet-derived growth factor, PDGF) leading to the inflammatory response by the recruitment and activation of macrophages and fibroblasts. Macrophages have a key role in phagocytosis of microrganisms and remnants of the ECM and in the secretion of wound healing cytokines such as PDGF, vascular endothelial growth factor (VEGF) and also transforming growth factor β (TGF-β) and basic and acidic fibroblast growth factor (FGF), which mediate the transition between inflammation and tissue repair. Fibroblasts are key cells in the formation of new tissue in the wound space. After migrating into the wound area, they synthesise new ECM with gradual replacement of the provisional matrix with a collagen matrix. Several matrix metalloproteases (MMPs) and tissue inhibitors of matrix metalloproteases (TIMPs) are expressed during wound

healing and are responsible for matrix remodelling by degrading existing matrix in/around the wound edge and by creating a path for cell migration while new matrix is deposited (McCawley and Matrisian, 2001). Matrix degradation is also thought to lead to the release of protein fragments that provide novel biological activities within the injury site important to facilitate repair (Davis et al., 2000). Fibroblasts subsequently assume a myofibroblast phenotype, express α-smooth muscle actin and initiate tissue compaction and wound contraction.

Endothelial cells, stimulated by the local secretion of angiogenic factors such as those belonging to the epidermal growth factor family (EGF) and the FGF family, are responsible for the neovascularization in the wounded area in the granulation tissue thus supporting the nutritional requirement of proliferating keratinocytes and fibroblasts. During the transition from granulation to scar tissue collagen is continuously remodelled with the formation of large collagen bundles and intermolecular crosslinks. Fibroblasts stop producing collagen and the granulation tissue is replaced by a scar, which is a relatively a-cellular matrix (Singer and Clark et al., 1999). This decrease in cellularity is mediated by apoptosis (Desmouliere et al., 1995). Many new blood vessels formed to sustain the granulation tissue are also eliminated as a result of apoptosis (Singer and Clark, 1999).

This brief description of wound healing demonstrates the wide array of molecular and cellular events underlying this process.

Tissue transglutaminase adds to the families of genes implicated in wound healing

In recent years, targeted disruption of genes encoding for growth factors and ECM components has revealed novel and specific roles in wound healing for a wide range of gene families. An example for this is demonstrated by targeted disruption of thrombospondin genes (TSP1 and TSP2), which has highlighted their function in the angiogenesis and inflammation steps of the wound healing process. Excisional wounds of TSP2-null mice are characterized by increased neovascularization and heal at an accelerated rate, suggesting a role for TSP2 in angiogenesis inhibition (Kyriakides et al., 1998; Agah et al., 2002). In contrast, absence of TSP1, a potent chemotactic factor for inflammatory cells, leads to delayed wound healing (Agah et al., 2002; Bornstein and Sage, 2002). Delayed skin wound healing has been observed in mice lacking FGF2 (basic fibroblast growth factor) and mice deficient in MMPs display impaired wound repair (McCawley and Matrisian, 2001). Delayed wound healing has been reported in TGF-β_1 deficient mice lacking T and B cells (Crowe et al., 2000). Recently, alteration of the FN alternative splicing in mice has demonstrated that while disruption of the FN gene is lethal in mice, animals devoid of EDA exon (extra domain A) display abnormal skin wound healing and both mice with complete exclusion or constitutive inclusion of the EDA exon have a shorter lifespan than wild-type mice, possibly because of defective wound repair (Muro et al., 2003).

A novel component of the cell/tissue response to cell damage and stress is tissue transglutaminase (tTG; TG2; EC2.3.2.13), a multifunctional protein which modulates cell-matrix interactions, tissue stability and a variety of cell functions (Aeschlimann and Thomazy, 2000; Griffin et al., 2002a; Fesus and Piacentini, 2002; Lorand and Graham, 2003). A definitive role for tTG in tissue repair, which is the main focus of this review, has been recently confirmed in tTG-deficient mice (Nanda et al., 2001; De Laurenzi and Melino, 2001) which presented with impaired skin wound healing (Mearns et al., 2002). Tissue transglutaminase is the first described member of the transglutaminase family, which so far includes nine members. Unlike other transglutaminases, tTG is characterised by unique structural features which leads to a wide range of biological activities and physio/pathological implications. Although some controversy and redundancy of information is present on the definitive functions of tTG, rapid advances have been made in the past few years and a triple mechanism of action for tTG (protein transamidation activity; GTPase activity; structural adhesion activity) can be proposed. tTG is characterised by a papain-like catalytic triad (Cys His Asp) responsible for a Ca^{2+}-induced transamidating activity which catalyses a range of tTG-mediated reactions leading to post-translational modifications of proteins, of which the cross-linking of proteins by ε-(γ-glutamyl)lysine bonds is probably the most frequent (Lorand and Graham, 2003). Integrated with the Ca^{2+}-regulated transamidase active site is a GTP binding and hydrolysis site, which is responsible for the G-protein/signal transduction function of tTG, which for this role is also known as Ghα. Binding of GTP negatively regulates the protein transamidation activity of tTG by inducing a conformational change that blocks the access of substrates to the transamidating active site (Liu et al., 2002). Reciprocally, the binding of Ca^{2+} inhibits the binding of GTP. Tissue transglutaminase is expressed ubiquitously and the expression level depends on the type of tissue. Typically, high levels of tTG can be found in cells naturally subject to insult such as endothelial cells and mesangial cells (Fesus and Thomazy, 1988).

tTG is localised in three major cell compartments (cytosol, plasma membrane and nucleus) and unlike other transglutaminases is secreted and deposited in the ECM. The mechanism of secretion is unusual because tTG lacks a signal peptide and is not secreted by a classical endoplasmic reticulum/Golgi-dependent mechanism. It is known that tTG secretion requires the active-state conformation of tTG (Balklava et al., 2002) and an intact N-terminal FN binding site (Gaudry et al., 1999b) but due to its atypical secretion mechanism, tTG is not efficiently released. However its release dramatically increases in situations of tissue damage and cellular stress (Upchurch et al., 1991; Johnson et al., 1999; Haroon et al., 1999) when it accumulates in the ECM initially in complex with FN. The protein cross-linking activity of tTG is tightly regulated both inside the cell, by the Ca^{2+}/GTP:GDP ratio and outside the cell by matrix binding and red-ox state of the Cys active site (Verderio et al., 2003; Cocuzzi and Chung, 1986). An *in situ* transglutaminase activity assay based on a small-size fluorescent primary amine substrate has clearly shown that tTG is in a catalytically active state while present at the cell surface. In contrast, the tTG transamidating activity would be mostly latent intracellularly (Smethurst and Griffin, 1995; Verderio et al., 1998). More recently, a cross-linking independent "structural" function of tTG has been found in the extracellular space by many authors (Akimov et al., 2000; Balklava et al., 2002; Verderio et al., 1993).

Due to its multiple activities and cellular distribution, tTG can potentially target the tissue repair process at multiple levels. The complex interrelations between the actions of tTG and the repair process are schematically depicted in Fig. 1 and described in the next sections.

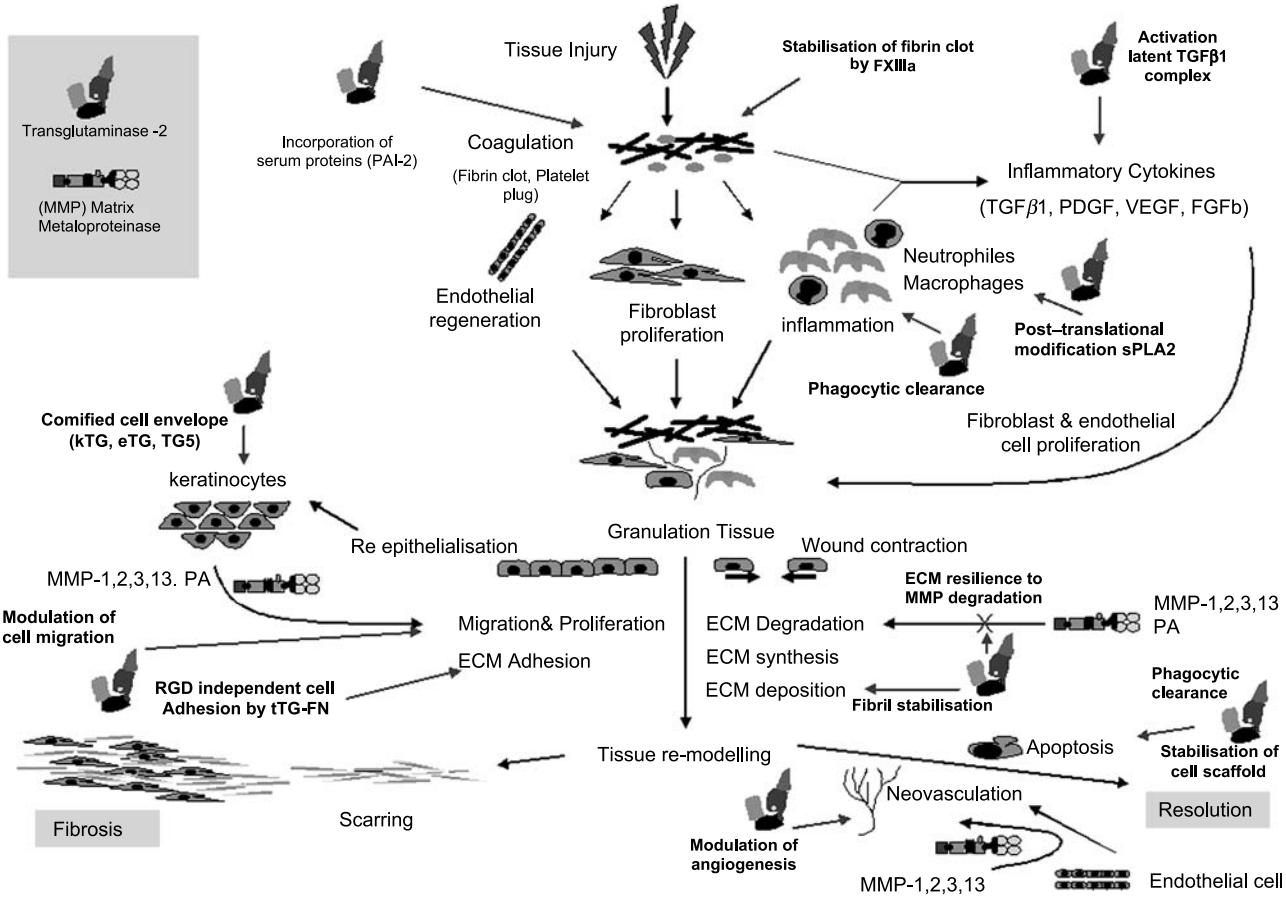

Fig. 1. Complex interrelations between the actions of tTG (tranglutaminase-2) and the repair process. The scheme depicts the phases of cutaneous wound healing, however most of the repair process applies to other tissues. Abbreviations: FXIIIa factor XIII subunit a; *kTG*, keratinocyte transglutaminase; *eTG*, epidermal transglutaminase; *TG5*, transglutaminase type 5 (Grenard et al., 2001a; Candi et al., 2003); *sPLA2*, secretory isoform of Phospholipase A2; *FN*, fibronectin; *ECM*, extracellular matrix; *MMP*, matrix metalloproteinase; *PA*, plasminogen activator; *PAI-2*, plasminogen activator inhibitor 2; *TGF-β* transforming growth factor β_1; *PDGF*, platelet-derived growth factor; *VEGF*, vascular endothelial growth factor; *FGF*, fibroblast growth factor; *DEJ*, dermo-epidermal junction

Secretion of tTG in response to tissue injury and stress

During tissue injury cells in the wound site are induced to synthesise and release molecules which participate in the regulation of the tissue injury response. These are typically ECM components such as osteopontin, SPARC, thrombospondins alternatively spliced FNs and collagen (Davis et al., 2000). Studies on skin by Bowness et al. (1988) reported increased transglutaminase activity during wound healing in rats. At the cellular level, Upchurch et al. (1991) demonstrated binding of endogenous tTG to the ECM following puncture wounding a fibroblast cell monolayer and persistence of tTG around the wound area for many hours afterwards. Haroon et al. (1999) reported not only an increase in tTG activity but also an increase in tTG expression by day 3 post-wounding following punch biopsy wounds in rats. Immunohistochemical detection of TG expression (with the monoclonal anti-tTG antibodies TG100 and Cub7402 at concentrations which are non reactive to factor XIIIa) and its isopeptide bond activity product (814 MAb CovalAb) during rat dermal wound healing showed that tTG is expressed and active in endothelial cells, macrophages and skeletal muscle cells in all stages of wound healing (Haaron et al., 1999).

Therefore tTG adds to the list of matrix components that are induced following tissue injury. Evidence is provided by the literature of regulation of tTgase expression by cytokines implicated in the repair process such as transforming growth factor β_1 (TGF-β_1), interleukin-6 (IL-6), tumor necrosis factor α (TNF-α) (George et al., 1990; Suto et al., 1993; Ikura et al., 1994; Kuncio et al., 1998). TGF-β_1 plays a central role in the wound response acting at a multiplicity of levels and influencing the inflammatory response, ECM deposition and remodelling and angiogenesis (Shah et al., 1999). IL-6 is an important inflammatory cytokine whose suppression in transgenic mice leads to impaired wound repair (Gallucci et al., 2000). TNF-α is mainly released by neutrophils and leads to pleiotropic expression of growth factors. Evidence for tTG regulation by cytokines is sustained at the molecular level by the isolation of the regulatory regions of the tTGase gene (Ritter and Davies, 1998). Our recent work suggests that cell stress resulting from a number of external stimuli can result in the regulated release of tTG from cells. For example, exposure of Swiss 3T3 fibroblasts to the mitochondrial dysfunction agent 3 nitropropionic acid (3-NP) leads to early stress induced release of tTG prior to release of the intracellular marker lactate dehydrogenase (Li et al., 2002).

We have also recently reported that many of the stress factors e.g. hyperglycaemia (Skill et al., 2003), acidosis (Johnson et al., 2002) and hypoxia (Fisher et al., 2002) associated with the progressive deterioration of kidney function and architecture can also lead to changes in cellular distribution of tTG in a number of different kidney tubular epithelial cell lines. As with 3-NP, induced release of tTG which is subsequently found at the cell surface and in the extracellular matrix appears to be a controlled and regulated process. Interestingly, in these tubular epithelial cell lines potential inducers of tTG such as TGFβ_1, TNFα, IL-1β_1, retinoic acid and dexamethasone fail to alter tTG expression (Johnson et al., 2002) raising questions as to which stress factors lead to cellular release of the enzyme. Studies in liver fibrosis suggest that NFKβ (Mirza et al., 1997) may be a key player but little of the upstream signalling was defined.

Role of tTG in the cell-extracellular matrix events important in wound repair

The entire tissue repair process is regulated by the interaction of cells with the surrounding ECM, thus ensuring cell adhesion, survival and proliferation (Sechler and Schwarzbauer, 1998; Singer and Clark, 1999).

For example the production of FN by fibroblasts along with its cell-surface receptors, is an essential step in the formation of new granulation tissue. FN participates in the regulation of the wound-repair response by providing a provisional matrix prior to collagen deposition which is essential for adhesion, migration and proliferation (Davis et al., 2000).

Biochemical observations first made by Lorand and confirmed by other authors have clearly demonstrated that FN has a high affinity binding site for tTG which would lie on the N-terminal gelating binding fragment of FN (LeMosy et al., 1992; Radek et al., 1993; Gaudry et al., 1999b). tTG appears as a globular protein bound to the N-terminal portion of FN, when visualised by rotary shadowing electron microscopy (LeMosy et al., 1992). Many of the extracellular roles of tTG which have implications in the early stages of wound repair involves its interaction with FN.

Our studies and those of other groups have confirmed that tissue transglutaminase is a FN-binding protein in cultured cells (Verderio et al., 1998; Gaudry et al., 1999) and it is able to modulate the FN matrix by forming non reducible Ca^{2+}-dependent ε-(γ-glutamyl)lysine cross-links (Jones et al., 1997; Verderio et al., 1998). Once released from cells, following cellular damage, inflamma-

tion or cell stress (Upchurch et al., 1991; Johnson et al., 1999; Haroon et al., 1999), tTG binds tightly to FN, as we have recently visualised by immunofluorescence and immunogold electron microscopy (Gaudry et al., 1999b; Verderio et al., 1998, 1999). Binding of FN to tTG protects tTG from proteolytic degradation (Belkin et al., 2001) and also leads to downregulation of tTG transamidating activity. Conversely, cell-surface tTG modulates and alters FN matrix stability by multimerisation following cell damage (Gross et al., 2003). It has also been proposed that by interacting with the gelatin binding domain of FN, tTG may cooperate with $\alpha_5\beta_1$ integrins in the assembly of the FN matrix (Akimov and Belkin, 2001a).

The cross-linking function of tTG in the extracellular matrix leading to ECM stabilisation/remodelling has been identified in a number of biological processes important for tissue repair (Aeschliman and Thomazy, 2000; see also section ''tTG and abnormal healing leading to fibrosis and scarring''). However an increasing number of reports indicate that the extracellular function of tTG is not solely dependent on its crosslinking activity, which may be downregulated by matrix binding (LeMosy et al., 1992; Verderio et al., 2003). tTG participates in cell matrix interactions which are fundamental in the tissue repair process such as cell adhesion, cell spreading and cell movement of fibroblasts. Rather than through increased matrix stability tTG can affect cell-matrix interactions either as an adhesion co-receptor of β_1 and β_3 integrins, or as an independent cell adhesion protein (Akimov et al., 2000; Gaudry et al., 1999a; Balklava et al., 2002; Belkin et al., 2001; Isobe et al., 1999). We have also proposed a mechanism whereby tTG function is strictly dependent on its specific association with matrix FN and in this complex tTG would support an RGD-independent cell adhesion process that is not linked to its transamidating activity (Verderio et al., 2003) (also see section ''Role of tTG in RGD-independent cell adhesion and cell survival'').

Role of tTG in matrix stabilisation

Cell proliferation, cell movement and cell interactions with matrix active sites depends on a regulated balance between matrix synthesis and matrix degradation. tTG-catalysed ε-(γ-glutamyl)lysine cross-links are stable to proteolytical and mechanical damage, therefore confer increased stability and resistance to degradation to the modified extracellular matrix proteins (Johnson et al., 1999).

Haroon et al. (1999) reported the increased expression of tTG found in endothelial cells and macrophages invading the fibrin clot results in the formation of transglutaminase-mediated cross-linking in both fibrin and the new granulation tissue during wound healing (Haaron et al., 1999). This finding is consistent with the observation that human umbilical vein endothelial cells (HUVEC) are a rich source of tTG, the synthesis of which is up-regulated by thrombin. (Auld et al., 2001). tTG retains activity in the ECM of HUVEC, contributing to the stability of the matrix (Mitchell et al., 2002). Factor XIIIa and possibly tTG are thought to contribute to the cross-linking of plasminogen activator inhibitor-2 (PAI-2) and α2-antiplasmin, which are inhibitors of fibrinolysis, to different lysine residues of fibrin/(ogen), thus further contributing to the stabilisation of the fibrin clot (Ritchie et al., 2000). In the epithelial layer tTG is expressed at the dermo-epidermal junctions, where it may serve to attach the epithelial layer to the dermo-epidermal junction (Ragunath et al., 1996). The ability of tTG to form non reducible multimers of extracellular matrix FN leading to its stabilisation (Martinez et al., 1994; Jones et al., 1997; Verderio et al., 1998; Gross et al., 2003) is also well demonstrated. Exposure of human dermal fibroblasts to non lethal ultraviolet doses (UV) results in increased tTG activity which does not require *de novo* protein synthesis and leads to multimerisation of FN into non reducible high molecular weight polymers. These data are consistent with a role for tTG in the rapid stabilisation of tissue following UVA damage (Gross et al., 2003; Nicholas et al., 2003).

Following wounding of a monolayer of Swiss 3T3 cells induced to overexpress tTG, a dramatic increase in tTG cross-linking activity could be measured at the edge of the wound bed by monitoring the incorporation of a fluorescent amine substrate for tTG. Following wounding massive intracellular crosslinking (detected by incorporation of labelled primary amine) was also evident in both Swiss 3T3 cells and in an endothelial cell line ECV304 which has high constitutive levels of tTG (Fig. 2). It is likely that this increased activity of tTG both in the intracellular and extracellular environment would lead to *in vivo* stabilisation of the wound area. Increased multimerisation by tTG may serve to stabilise the matrix and/or increase matrix valency thus enhancing cell-matrix interactions such as cell adhesion and migration in the wound area to promote wound healing.

Other members of the TG family which are likely to contribute to the stabilisation of the provisional matrix are factor XIIIa, and also epidermal transglutaminase, which is expressed in keratinocytes and other epithelial-derived cells (Lorand and Graham, 2003). However, unlike factor

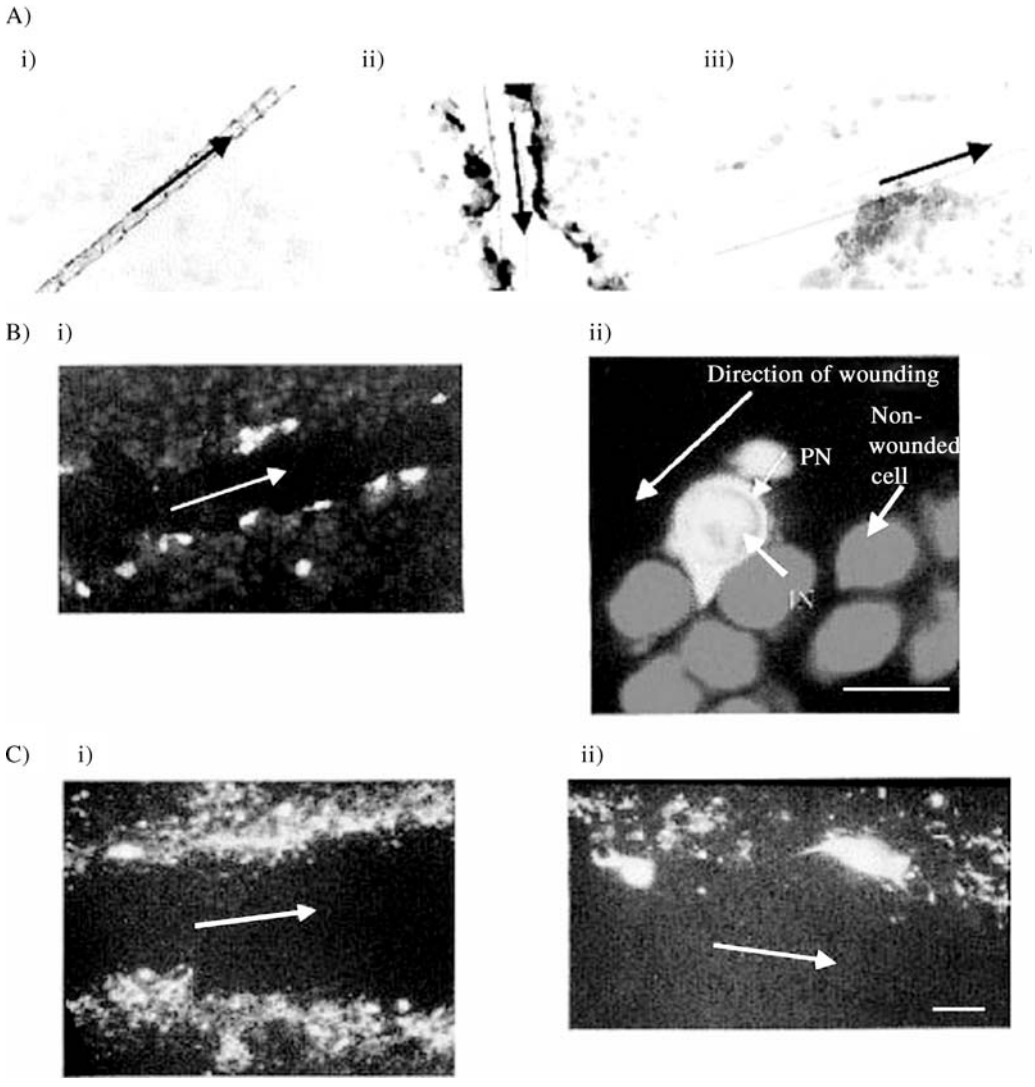

Fig. 2. Time course of tTG activity in wounded cell monolayers of Swiss 3T3 cells. **A** ECV 304 cells were wounded using a sterile plastic pipette tip. They were then incubated in the presence of the primary amine tTG substrate biotinylated cadaverine (BTC) for 20 min and detected by DAB stain and visualised using light microscopy. Control, no BTC(panel i). +BTC (panel ii). +BTC and 5 mM putrescine (panel iii). Cells were counterstained with propidium iodide. **B** ECV 304 cells wounded as described above and then incubated in the presence of BTC and incorporation revealed using streptavidin-FITC. ×10 magnification using conventional fluorescence microscopy (panel i). ×63 magnification using confocal laser microscopy, showing a 1 μm section through the middle of the cells (panel ii). Cells are counterstained with propidium iodide. **C** Swiss 3T3 cells (clone TG3) induced to overexpress tTG and wounded as previously described in the presence of the primary amine tTG substrate fluorescein-cadeverine and then examined after 20 min at ×10 magnification (panel i) and ×40 magnification (panel ii) using a Leica TCSNT confocal laser microscope. The micrographs shown are 1 μm sections through the middle of these cells. Arrows indicate the direction of wounding, and scale bars represent 10 μm. Taken from Nicholas et al. (2003) with permission of authors and permission of the Biochemical Society

XIIIa, tTG does not require thrombin activation and therefore could continue to serve as a matrix stabiliser in the absence of thrombin when the latter is removed from the site of injury.

We have reported that increased tTG expression in Swiss3T3 fibroblasts using a tetracycline-regulated system, leads to an increased pool of FN fibrils compared to non-induced cells expressing low background level of tTG, suggesting that modulation of tTG expression affects the insoluble FN fraction. The increased FN deposition in this cell model may result from increased matrix stabilisation, since Swiss3T3 cells overexpressing TG display a fourfold increase in $\varepsilon(\gamma$-glutamyl)lysine crosslink when compared to the noninduced cells, which is likely to be mostly formed extracellulary (Verderio et al., 1998).

During the transition from granulation tissue to scar tissue remodelling plays a key role and depends on the balance between the synthesis and degradation of collagen (Singer

and Clark, 1999). Collagen remodelling leads to the formation of large bundles with the creation of new intermolecular crosslinks, which give tensile strength to the scar. We have demonstrated using *in vitro* studies that collagen I when cross-linked either in the presence or absence of FN by tissue transglutaminase leads to increased stability to MMP-1 (Johnson et al., 1999). Apart from collagen 1, tissue transglutaminase has been shown to cross-link a number of different types of collagen (II, III, V, XI) and other ECM proteins (Aeschlimann et al., 1996; Aeschlimann and Thomazy, 2000; Nomura et al., 2001; Griffin et al., 2002a; Lorand and Graham, 2003).

Crosslinking-independent roles of tTG in cell migration

FN and fibrin provide a provisional matrix for the concomitant migration of inflammatory cells, fibroblasts and endothelial cells (Singer and Clark, 1999).

The contribution of tTG to cell adhesion and migration has now been defined in a number of different cell systems. For example, induction of tTG expression in Swiss 3T3 fibroblasts transfected with the catalytically active and inactive tTG (cys^{277} ser mutant) under control of the tet regulatory system (Verderio et al., 1998) is accompanied by a decrease in cell migration on FN (Balklava et al., 2002) when cell motility was assayed by measuring outward cell migration from an agarose droplet on FN (Balklava et al., 2002). This data suggest that the cross-linking activity of tTG is not responsible for the observed effect of tTG on cell motility.

In agreement with these data, assays of *in vitro* wound closure tests revealed a slower rate of migration of tTG transfected Swiss3T3 fibroblasts when induced to over-express tTG compared to cells expressing a low background level of tTG (Fig. 3). In addition the ability of anti-tTG monoclonal antibody Cub7402 to reduce cell migration in a dose-dependent manner (Balklava et al., 2002) indicates that cell-surface tTG is an important component in the migration of cells. Indeed incubation of cells with Cub 7402 leads to loss or reduction of cell attachment in these (Verderio et al., 1998) and other cell types (Jones et al., 1997; Heath et al., 2001), in a similar manner as described for cells incubated with antibodies directed against cell surface integrin receptors β_1 and α_5 (Fogerty et al., 1990). In a different cell system Akimov and Belkin

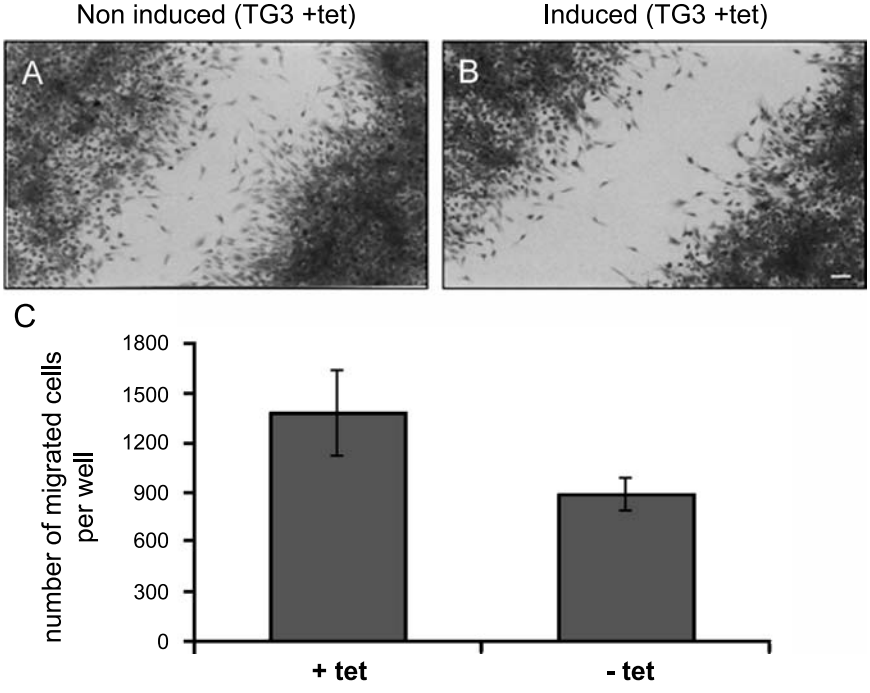

Fig. 3. *In vitro* migration analysis of transfected Swiss 3T3 fibroblasts (clone TG3). Fibroblasts non-induced (**A**) and induced (**B**) to overexpress tTG by withdrawal of tetracycline from the medium, were cultured in DMEM with 10% FCS until confluency. *In vitro* wound clousres test were performed by introducing a wound in the monolayers with a plastic tip (diamter 1 mm). The monolayers were then covered with fresh medium and incubated for a 20-h period. Following fixation of cells and staining with crystal violet the number of cells migrated in the cleared area was assessed by examining cells "in blind". Bar, 20 μM. Quantifications of the migrated cells (**C**) showed that cells over-expressing tTG migrated into the wound bed significantly less ($p < 0.05$) compared with non-induced controls. Results are from a typical triplicate experiment

(2001a) have demonstrated that tTG, which is expressed on the surface of monocytic cells, is also involved in the adhesion and migration of monocytic cells on a FN matrix. During the differentiation of monocytes into macrophages tTG expression is increased.

These *in vitro* models are likely to represent only a fraction of a more complicated mechanism *in vivo*. However, as previously referred to, initial *in vivo* studies on wound healing using punch biopsy-induced skin lesions appear to show a significant delay in wound closure in tTG-deficient mice compared to control wild type littermates (Mearns et al., 2002).

Role of FN-bound tTG in mediating RGD-independent cell adhesion and cell survival

During tissue injury when the composition of the ECM and its molecular structure are altered in several significant ways, modulation of cell adhesion as a result of matrix alterations, may be an important step in wound-repair responses, which involves the dynamic interactions of multiple cells types with the extracellular matrix (ECM) (Davis et al., 2000).

In a recent study (Verderio et al., 2003) we demonstrated that matrices of FN in complex with tTG, prepared either *in vitro* using purified proteins, or by cell-secretion forming a physiological ECM (tTG-FN), have a distinctive adhesive role. In response to tTG-FN, various cell types (e.g. fibroblasts, osteoblasts and endothelial-like cells) could largely restore loss of cell adhesion following inhibition of the classical FN ArgGlyAsp (RGD)-dependent adhesion pathway mediated by $\alpha_5\beta_1$ integrin receptors. In contrast, the simple binding of purified guinea pig liver tTG to either tissue culture plastic or the gelatin binding domain of FN, which contains the tTG binding site, did not enhance cell adhesion, which requires the specific complexation of tTG with FN. This matrix complex was sufficient to support the formation of focal contacts in the presence of RGD peptide, which are visualised by indirect fluorescent staining of vinculin in Fig. 4, and

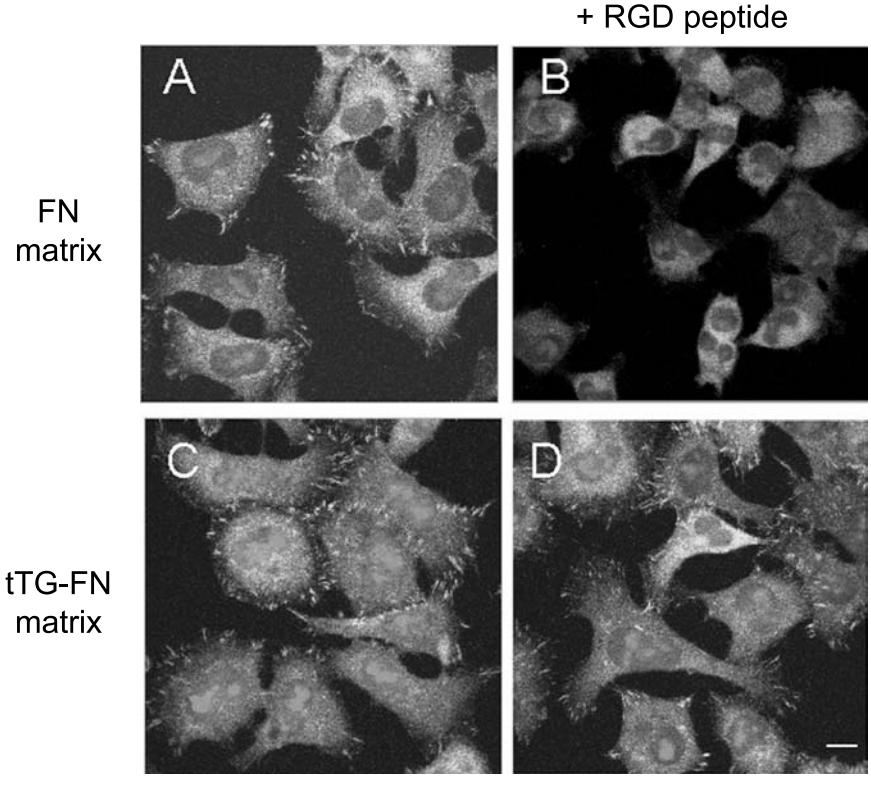

Fig. 4. Confocal laser fluorescence microscopy of RGD-independent focal adhesion in response to tTG-FN. Primary human osteoblasts were seeded in 0.79 cm^2-wells of chamber slides (8×10^4/well) previously coated with FN and FN with immobilised tTG as described (Verderio et al., 2003) and allowed to adhere for ~20 minutes. Focal adhesions formed by cells on FN in the presence (**A**) or absence of RGD peptide (**B**) and on tTG-FN with (**C**) or without (**D**) RGD peptide, were detected on fixed and pereabilised cells by indirect immunofluorescent staining for vinculin, using a monoclonal anti-vinculin antibody followed by secondary antibody conjugated to FITC. After nuclear staining with propidium iodide, cells were imaged by confocal fluorescent microscopy using a Leica TCSNT confocal laser microscope system, equipped with an argon/krypton laser adjusted at 488 nm for fluorescein excitation. Bar, 10 μM

the assembly of associated actin stress fibers (Verderio et al., 2003). Inhibition of RhoA by C3-exotransferase completely blocked RGD-independent formation of actin stress fibers by tTG-FN. A PKCa inhibitor (Gö6976) (Gschwendt et al., 1996), negatively affected RGD-independent cell adhesion to tTG-FN, suggesting the involvement of PKCa. Analysis of focal adhesion kinase (FAK) activation implicated an enhanced tyrosine phosphorylation of FAK during this process.

We have no evidence so far of a direct activation of FAK by PKCα in response to tTG-FN. Recently it was shown that activation of RhoA stimulates FAK Tyr_{397} phosphorylation and that PKC activity is not involved in this process (Wilcox-Adelman et al., 2002). However it was also proposed that both pathways, PKC and Rho are separately required for full cell adhesion (Woods and Couchman, 2001 and reference therein).

At this stage it is still not entirely clear how these intracellular mediators convey signals from the extracellular tTG-FN.

Cell adhesion to tTG-FN is not linked to the intrinsic tTG ability to modify the FN matrix by calcium-dependent transamidation, which is consistent with the previously described transamidating-independent role for tTG in cell-matrix interactions (Belkin et al., 2000; Balklava et al., 2002). Treatment of HOB cells with heparitinase, an enzyme that catalyses the eliminative cleavage of heparin and heparan sulfate, greatly diminished the RGD-independent adhesion in response to tTG-FN, suggesting that cell-surface HSPGs may mediate RGD-independent cell adhesion to tTG-FN.

It is accepted that the RGD-mediated cell adhesion plays a central role in cell survival and synthetic peptides containing the RGD motif induce apoptosis in many cell types, by acting as competitive inhibitors of FN-integrin interaction and activators of caspase 3 (Hadden and Henke, 2002; Buckley et al., 1999).

The observation that tTG-FN can rescue tTG-null primary dermal fibroblasts from detachment-induced apoptosis mediated by RGD peptide is consistent with the finding that RGD-independent cell adhesion to tTG-FN is linked to the intracellular cell survival kinase FAK (Frisch et al., 1996; Frisch and Screaton, 2001).

Such a complex of tTG and FN may be necessary to ensure adhesion-mediated cell survival in situations of cell wounding or cell stress, where the increased expression of matrix-degrading metalloproteinases triggered by the inflammatory response, would lead to fragmentation of the ECM, disruption of cell adhesion-mediated integrin-signalling and finally apoptosis (Kapila et al., 1999).

Role of tTG in the inflammatory response

As outlined earlier, thrombus formation re-establishes hemostasis after tissue injury and provides a provisional ECM for cell migration. Trapped platelets within the clot trigger an inflammatory response and secrete wound healing factors such as PDGF that attract macrophages and fibroblasts. Much attention has focused on macrophages, which mediate the transition between inflammation and repair, by the secretion of wound healing mediators such as PDGF, VEGF and other cytokines such as those of the TGF-β and FGF families (Singer and Clark, 1999). The tTG antigen was found to be particularly expressed in macrophages, adjacent to the re-epithelialization zone and in the provisional fibrin matrix during rat dermal wound healing (Haaron et al., 1999).

By using mice deficient in tTG, Szondy et al. (2003) have recently found that lack of tTG in macrophages prevents efficient phagocytosis of dead cells. The phagocytosis of apoptotic cells was defective in the thymus of TG2-deficient mice after induction of apoptosis by either a dose of anti-CD3 monoclonal antibody, a dexamethasone-acetate injection or gamma irradiation. The defect in clearance of dead cells by macrophages leading to increased inflammation was related to the impaired activation of TGF-β_1, which is specifically released by macrophages on recognition of dead cells and play an important function in downregulating the inflammatory response (Szondy et al., 2003).

Crosslinking of intracellular proteins by tTG (see Fig. 2), which occurs if the insult produces a loss in calcium homeostasis (Verderio et al., 1998; Johnson et al., 1998; Nicholas et al., 2003), may also be significant to prevent leakage and lysis of dying cells, thus maintaining their structural integrity and the integrity of the tissue within which the damaged cells are dying thus further containing the inflammatory response (Nicholas et al., 2003).

Inflammation occurs as a defence response to physical damage. The initial local vasodilation and vascular permeability is followed by the release of arachidonic acid from the cell membrane by the enzyme phospholipase A2, which is the rate limiting step in the biosynthesis of eicosanoids by cyclo-oxygenase (COX). The levels and activities of these lipid mediators have been associated with the regulation of the inflammatory response and a number of pathological states including asthma, pulmonary fibrosis, as well as other inflammatory lung diseases. tTG activity is found to be upregulated in various inflammatory conditions and in diseases which are characterised by mucosal inflammation, such as celiac disease, Crohn's disease,

ulcerative colites (Lorand and Graham, 2003). The activity of sPLA2s (secretory isoforms of phospholipase A2), a membrane protein which releases arachidonic acid during inflammation, is enhanced by tTG either through the formation of an isopeptide bond within sPLA2 or through its polyamination (Cordella-Miele et al., 1990, 1993). Sohn et al. (2003) have recently shown that new chimeric peptides which are derived from pro-elafin and antinflammins, can inhibit sPLA2, tTG activity and tTG-mediated modification of sPLA2 and display strong *in vivo* anti-inflammatory activity. In a transgenic mouse model overexpression of tTG in ventricular myocytes under the control of a alfamyosin heavy chain promoter, leads to upregulation of cycloxygenase-2 (COX-2), thromboxane synthase (TxS) and results in cardiac failure (Zhang et al., 2003).

Role of tTG in neovascularisation

Neovascularization is necessary to support the new granulation tissue, which form and invade the wound space after injury. It is a complex and dynamic process involving stimulation of endothelial cells by both serum factors (VEGF, FGF, TGF-β) and the ECM environment in the wound bed. The migration of endothelial cells and the formation of new vessels is affected by the composition of both the granulation tissue and the endothelial basement membrane (Zhang and Kirsner, 2003).

Following injury tTG is externalised, binds to the ECM and affects both the stability and structure of the ECM by virtue of its dual cross-linking and structural role. By modulating matrix storage of latent TGF-β_1, tTG participates in the activation of this important wound healing cytokine (Taipale et al., 1994; Kojima et al., 1993; Nunes et al., 1997; Verderio et al., 1999). All these tTG-mediated events are likely to influence angiogenesis during wound repair. Haroon et al. (1999) have found that recombinant tTG enhances blood vessel length density if applied topically in an *in vivo* dorsal skin flap window chamber, suggesting the implication of tTG in promoting angiogenesis in wound repair.

In contrast if tTG is administered in the matrix in a pre-reduced activated state at sustained high doses (Patent Application PCT/GB01/03574), thus overtaking its physiological control mechanisms, it can suppress endothelial tube formation without cell toxicity in an *in vitro* co-culture angiogenesis model (Griffin et al., 2002b). Following this sustained tTG-treatment, the ECM embedding the capillary vessels displays a high level of transglutaminase activity (measured *in situ* via fluorescein-cadaverine incorporation) and increased matrix stability.

Interestingly gene array analysis of differential gene expression during human capillary morphogenesis in 3D collagen matrices has classified tTG among the genes which are regulated during this process. Indeed tTG was found to be remarkably downregulated, approximately 4-fold at 8 hour and 10-fold at 24 and 48 hour of endothelial cell cultures in 3D collagen matrix (Bell et al., 2001), thus confirming that lowered levels of tTG expression are required during the initial stages of angiogenesis.

tTG and abnormal healing leading to fibrosis and scarring

As outlined at the start of this review wound healing is characterised by three interrelated and overlapping phases: inflammation, tissue formation/stabilisation and tissue remodelling. However, once these phases are completed there must be resolution of the inflammatory response, clearance of the inflammatory cells and a return to normal ECM homeostasis. If the insult continues (e.g. diabetes, chronic infection, genetic disease, toxic exposure, chronic inflammation e.g. as a result of auto immunity) or alternatively the termination/resolution of part of the wound response fails to initiate correctly, a period of chronic wounding ensues that can lead to progressive scarring and fibrosis. In the major organs such as liver, heart and kidney this progressive scarring is the major cause (over 95%) of organ dysfunction and failure. The mechanisms underlying progressive scarring have been the subject of intensive research over several decades, yet there is still no effective therapy for this treatment.

The observation that tTG may be involved in progressive scarring was first reported in the lung (Griffin et al., 1978), although similar observations have subsequently been reported in liver (Mirza et al., 1997; Grenard et al., 2001b), heart (Small et al., 1999), vasculature (Bowness et al., 1994) and perhaps most extensively in the kidney (Johnson et al., 1997) (see Fig. 5 and Fig. 6). In 1978 using a paraquat induced model of pulmonary scarring we reported an upregulation of TG activity (Griffin et al., 1978). In a series of studies by Bowness and colleagues (Bowness et al., 1987, 1989, 1994; Bowness and Tarr, 1990) increased TG found in atherosclerotic plaques correlated with increases in the ε-(γ-glutamyl)lysine cross-link. TG mediated crosslinking was associated with stabilisation of the collagen III – lipoprotein interface surrounding the sclerotic plaque. Later Mirza and colleagues (1997) reported increased tTG in hepatic fibrosis with mRNA analysis showing an upregulation of tTG in most liver cell types. In later studies Small and

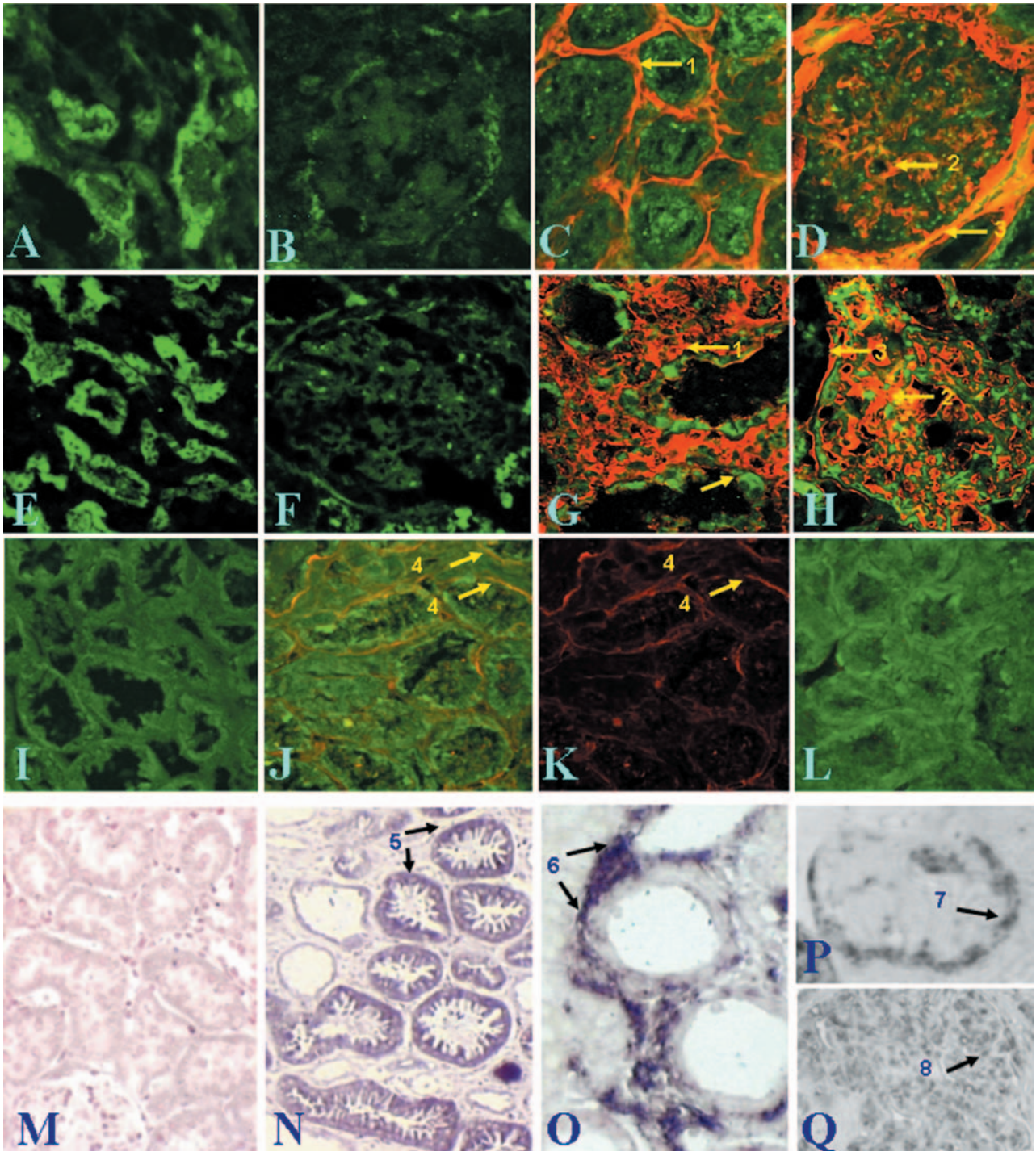

Fig. 5. Involvement of tissue transglutaminase in renal scarring. Panels **A**–**D**. Tissue Transglutaminase staining (red) in Normal Glomruli (**A**) and cortical tubulointerstitium (**B**) compared to that in renal scarring resulting from Focal Segmental Glomerulosclerosis. Arrow 1 indicates increased staining in the expanded tubulointerstitium, arrow 2 the mexangial matrix/glomerular basement membrane and arrow 3 periglomerular. Panels **E**–**F** $\varepsilon(\gamma$-glutamyl)lysine staining (red) in biopsies from implanted allgrafts (Glomeruli (**E**) and cortical tubulonerstitium (**F**)) compared to that in scarred grafts resulting from chronic rejection. Arrows indicate as in panels A–D. Panels **I**–**L**. Transglutaminase *in situ* activity (red) in normal rat kidney (**I**) and that from the $5/6^{th}$ subtotal nephrectomy model of renal scarring (**J**–**L**). Panel K excludes autofluorescent emissions (green) showing renal morphology. Arrow 4 shows activity is strongest peritubular in the tubular basement membrane and that tTg in the expanded ECM loses most of its activity. Panels **M**–**Q**. Tissue tranglutaminase *in situ* hybridisation (black) in normal rat kidney (**M**) and that from a $5/6^{th}$ subtotal nephrectomy model of renal scarring (**N**,**O**). Arrow *5* shows that proximal tubular cells are the predominant source of tTg in renal scarring, although arrow *6* indicates isolated patches of interstitial cells (Macrophages or myofibroblasts) are also able to synthesis tTG. Panel P shows a glomeruli from a patient with crescentic nephritis with tTG synthesis within the scarring crescent (arrow *7*) (myofibroblasts) and panel **Q** shows mesangial cell synthesis in a patient with mesangial proliferative glomerulonephritis. (Adapted from Johnson et al., 1999; 2003a with permission of authors and the American Society of Nephrology)

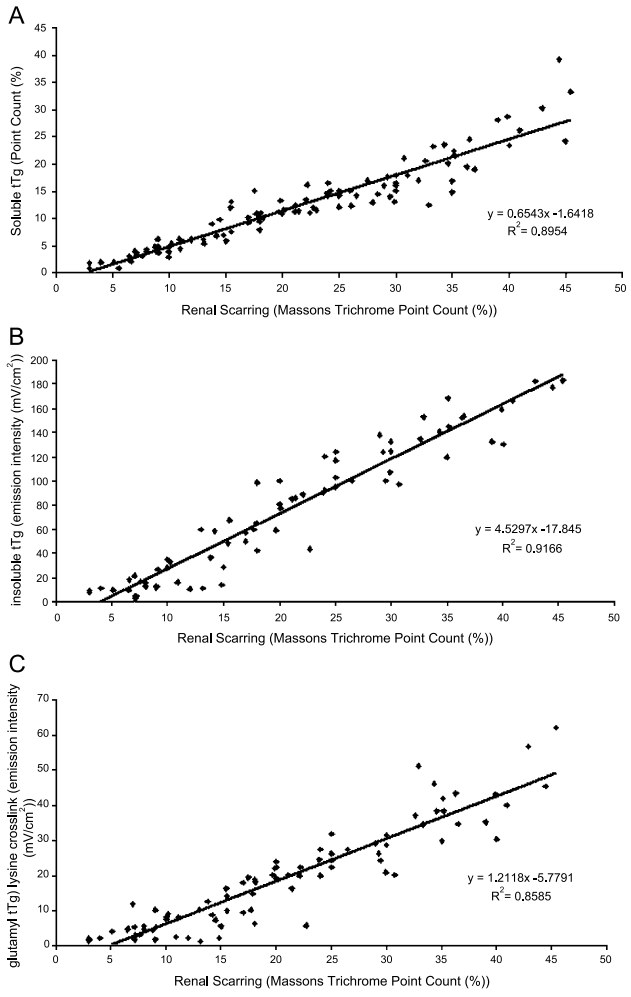

Fig. 6. Correlation of tissue transglutaminase and protein crosslinking to renal scarring. Quantification of staining for soluble tTG (**A**), insoluble tTG (**B**) and $\varepsilon(\gamma$-glutamyl)lysine staining (**C**) in 136 renal patients from a range of initial aetiologies correlated to the level of renal scarring as assessed by point counting of Masson's Trichrome stained sections. Insoluble tTG and $\varepsilon(\gamma$-glutamyl)lysine staining where measured using emission intensities from confocal microscopy, whereas soluble tTG was assessed by point counting

colleagues (Small et al., 1999), using a transgenic mouse with enhanced cardiac tTG expression showed that those animals developed cardiomyopathy. In the kidney, we first reported changes in TG in tubular epithelial cells in renal scarring in a subtotal nephrectomy model that led to increased ε-(γ-glutamyl)lysine levels found predominantly in the tubulointerstitium (Johnson et al., 1997). Later studies demonstrated that this increase was due to an elevated synthesis and cellular export of tTG by these tubular cells (Johnson et al., 1999). Similar studies in a streptozotocin model of early diabetic nephropathy further demonstrated the presence of increased levels of ε-(γ-glutamyl)lysine that were similarly located in the interstitial space, but also found in the glomerular basement membrance and in the expanding mesangial matrix with the mesangial cells being the primary source of tTG in the glomeruli (Skill et al., 2001). This study was particularly interesting since it showed that increased levels of tTG could be exported to the ECM independently of changes in tTG synthesis. More recently, we have been able to expand observations of tTG in kidney scarring to the clinical situation whereby 136 renal biopsies with varying degrees of scarring from a range of original etiologies were studied (Johnson et al., 2003a). This showed that changes in tTG and ε-(γ-glutamyl)lysine correlated exceptionally well with the degree of scarring and that changes were independent of the original disease.

The major problem with these changes observed for tTG *in vivo* in aberrant wound healing is that the insult is never removed, therefore the key question is whether these prolonged increases in tTG is one of the major causes in switching normal wound healing to pathological scarring or whether they are an attempt to minimise damage from a continuing insult. In order to address these issues we have recently undertaken pilot studies using irreversible site directed inhibitors of tTG in the subtotal nephrectomy model of renal scarring using direct intrarenal delivery from an implanted osmotic minipump (Johnson et al., 2003b). Our preliminary data indicate that inhibition of TG activity from the initiation of the insult preserves both renal architecture and function and that the degree of scarring (including deposited collagen) is vastly reduced. Interestingly however, if one over inhibits TG activity this leads to an extensive infiltration by monocytic derived inflammatory cells further suggesting that TG may have a role in modulating the immune response. The actual mechanism as to how inhibition of TG leads to a reduction in scarring which is essentially a reduction in collagen deposition following tissue remodelling still remains an open question. Evidence suggests that the storage of latent TGF-β_1, through latent TGF-β binding protein 1 (LTBP-1) in the ECM (Taipale et al., 1994) is an important early step in the proteolytic activation of TGF-β_1. As previously eluded to, LTBP-1 is a substrate for tTG *in vitro* (Nunes et al., 1997) and in cells in culture (Verderio et al., 1999), and tTG may mediate both matrix storage and activation of TGF-β_1. Both excess TGF-β_1 and increased expression of tTG have been implicated in a number of fibroproliferative conditions (Griffin et al., 1979; Mirza et al., 1997; Johnson et al., 1997). Hence reducing tTG activity should in theory reduce the levels of TGF-β_1 thus reducing collagen deposition by an indirect mechanism (see Fig. 7).

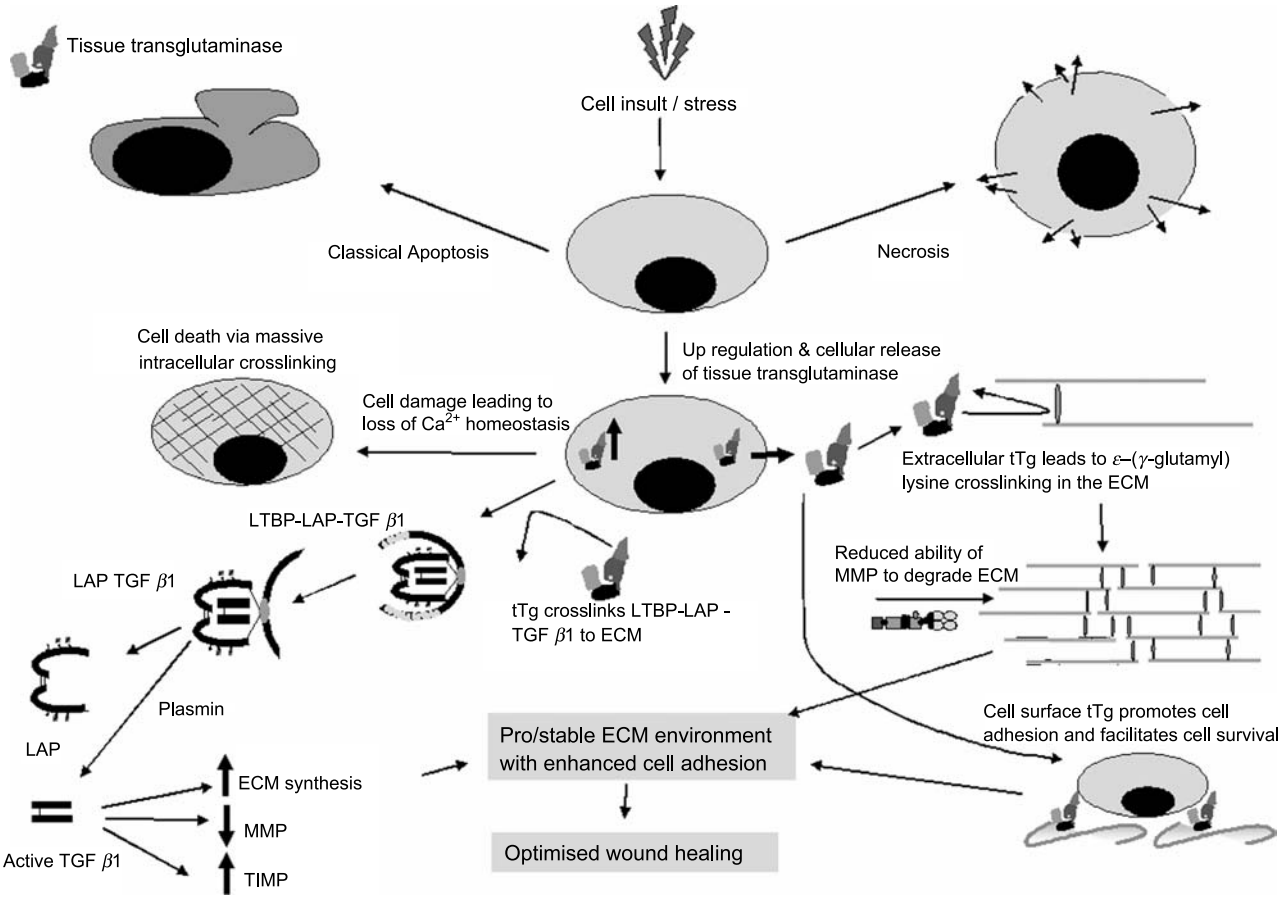

Fig. 7. Importance of tTG in the maintenance of tissue integrity following cell stress/injury. Tissue TG is normally in the extracellular matrix in relatively low amounts depending on the tissue. Following stress or insult, up-regulation of tTG often occurs, resulting in further enzyme externalized into the matrix. Insult leading to cell damage can also lead to increased tTgase leaking into the matrix. This is accompanied by the massive intracellular cross-linking of the tTgase containing dying cells following loss of Ca^{2+} homeostasis. Once increased in the matrix, the enzyme has both direct and indirect effects on the matrix, either through direct protein cross-linking leading to matrix stabilization or indirectly via the activation of matrix-bound TGFβ_1 leading to matrix deposition. Matrix-bound tTG can also act as an independent cell-adhesion protein when bound to fibronectin preventing cell death by anoikis. The end result is would healing and maintenance of tissue integrity. Abreviations: *tTG*, tissue transglutaminase; *MMP*, matrix metalloproteinase; *TIMP*, tissue inhibitor of matrix metalloproteinase; *LTBP*, latent TGFβ_1 binding protein; *LAP*, latency associated peptide

An alternative mechanism is that tTG mediated crosslinking of collagen and/or associated proteins has a direct effect on their deposition as previously eluded to earlier in this chapter.

In 1995 Kleman and colleagues (1995) using a rabhydosarcoma cell line demonstrated that tTG was able to cause lysyl oxidase independent crosslinking of collagen V and XI leading to stabilisation of the fibrillar structure. Although similar studies have not been demonstrated for other collagen types the ability of tTG to crosslink collagen fibrils in the terminal maturation step of collagen deposition, thought to be the preserved role of lysyl oxidase, suggested that tTG might be able to modify the rate at which collagen is deposited. Such a step could be crucial in the scarring process since upto 95% of the collagen molecules which are normally produced in massive excess are degraded prior to their deposition (Mays et al., 1991). Hence any step which tips the balance towards matrix deposition is likely to have a major influence on ECM homeostasis.

If excessive crosslinking does occur in the ECM during progression of the diseased state, then by definition this makes qualitative changes to the matrix. This may not only alter some of the properties of the ECM, but may alter its resilience to enzymatic decay. There are several enzyme systems involved in the breakdown of the matrix, with plasmin, cathepsins and the matrix metalloproteinases (MMP) being the major players. Of these, the 18 membered MMP family is the most prominent, with sub

classes of MMPs such as the gelatinases, stromolysin and interstitial collagenases targeting specific classes of ECM proteins. As referred to earlier, Kleman et al. indicated that tTG caused intramolecular crosslinking within the collagen V & XI fibrils in the N and C termini of the collagen molecules. Interestingly this is the same region in which interstitial collagenases such as MMP 1 and MMP 8 target to excise the lysyl oxidase catalysed pyridinoline/pyrrole trivalent crosslinks that stabilise the collagen fibril (Batge et al., 1990; Wu et al., 1990; Hasty et al., 1993). Therefore if $\varepsilon(\gamma$-glutamyl)lysine crosslinking occurred adjacent to the pyridinoline/pyrrole crosslinks then the removal of these lysyl oxidase catalysed bonds may not be sufficient to solubilise the collagen molecule essential for matrix turnover.

To test this hypothesis we initially employed a modification of the collagen fibril assay where isolated collagen 1 was formed into fibrils and crosslinked with tTG before exposure to MMP 1. tTG crosslinked collagen 1 was 3× less susceptible to the action of MMP-1 than a normal collagen fibril, and even less susceptible if FN is crosslinked to the collagen fibril by tTG (Johnson et al., 1999).

In a more recent study we have added exogenous tTG to cultures of human dermal fibroblasts to mimic the release of tTG that occurs following exposure to damaging agents such as prolonged exposure to UVA. Our results indicate that increased levels of tTG that might be deemed pathological lead to an increase in collagen deposition and a slowed rate of collagen turnover which would contribute to the scarring process consistent with that of skin ageing induced by solar radiation (Gross et al., 2003). Our preliminary studies with kidney tubular epithelial cells also indicate that increased expression of tTG in these cells brought about either via transfection or by exposure to high glucose levels typical of those found in diabetic nephropathy leads to changes in collagen deposition that may be independent of TGF-β_1 levels.

Hence if tTG is able to enhance the rate of ECM deposition and/or restrict the rate of ECM decay as our preliminary data suggests, then overexpression and cellular release of the enzyme would promote ECM accumulation leading to rapid wound closure and maintenance of tissue integrity. If however, the insult is prolonged then the accumulation of tTg in the ECM becomes pathological leading to excessive scar formation and fibrosis. These observations are consistent with and give further clarification to earlier findings that tTG activity is increased in hypertrophic scarring, resulting in excess-healing and accumulation of ε-(γ-glutamyl)lysine cross-links to form insoluble collagen matrices. Importantly the observation that this condition could be inhibited by 50 mM putrescine, a competitive primary amine substrate of transglutaminase that would reduce crosslinking, led to investigating the clinical effect of 50 mM putrescine in a eutectic vehicle (Fibrostat) which was studied in phase II clinical trial (Dolynchuk et al., 1994, 1996). The compound has so far reached Phase IIb clinical trials and represents a unique topical pharmaceutical preparations with an active pharmaceutical ingredient that reduces hypertrophic scarring.

Given the strong affinity of tTG for FN (Jeong et al., 1995) and the high affinity of FN for other ECM proteins, it is possible that tTG may also play a pivotal role in the cross-linking of other wound healing factors to the ECM, thus providing a further means of regulating the wound response.

Thymosin Beta 4 (Tβ4) is the most abundant member of a family of highly conserved and extremely water-soluble 5 kDa polypeptides; it is expressed in most cell types and is regarded as the main intracellular G-actin sequestering, regulating the growth and differentiation of actin and playing a role in wound healing (Malinda et al., 1999). There is increasing evidence for extracellular functions of Tβ4, which increases endothelial cell adhesion and migration, stimulates angiogenesis and down regulates a number of inflammatory cytokines, and it was recently found that tissue transglutaminase can cross-link Tβ4 to proteins such as fibrin and collagen (Huff et al., 1999, 2002). After activation of human platelets with thrombin, Tβ4 is released and cross-linked by transglutaminase (factor XIIIa), thus accumulating at sites of clots and tissue damage, where it may contribute to wound healing, angiogenesis and inflammatory responses (Huff et al., 2002).

Tβ4 represents a novel class of drugs in development to accelerate the healing process, speed the growth of blood vessels, and decrease inflammation (Philp et al., 2003). A Phase 1 clinical trial with a chemically synthesized copy of Tβ4 has recently begun in 2003.

Conclusions

In summary (see Fig. 7), following tissue injury, accumulation of tTG in the matrix of the surrounding area appears to lead to the intermolecular crosslinking of many ECM components (especially collagens and fibronectin), probably leading to their accelerated rate of deposition. During normal wound healing tTG transamidating activity is likely to be rapidly downregulated by binding to matrix proteins such as FN and by the oxidizing extracellular environment. Importantly, binding of tTG to FN may also

form a complex with novel pro-survival characteristics which mediates cell signalling through the HSPG class of transmembrane receptors. In addition, extracellular tTG may control the storage of latent TGF-β_1, thus influencing the activation of TGF-β_1, which is not only a major player in the inflammatory response, but also crucial in regulating ECM homeostasis via its regulation of MMP and TIMP synthesis. In the initial insult to a tissue loss of Ca^{2+} homeostasis in cells could also promote rapid intracellular crosslinking by tTG providing an alternative fast mechanism to apoptosis for preventing loss of intracellular components and thus limiting the inflammatory response.

If however, the insult to the tissue is maintained leading to massive accumulation of both intracellular and extracellular levels of tTG then the preservation of tissue integrity leads to a pathological event promoting ECM accumulation and excessive tissue scarring.

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