Increased TG2 expression can result in induction of TGFβ1 causing increased synthesis and deposition of matrix proteins which can be regulated by nitric oxide.

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Running Title: TG2 mediated NO regulated extracellular matrix biosynthesis
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In fibrotic conditions increases in TG2 activity has been linked to an increase in the deposition of extracellular matrix (ECM) proteins. Using TG2 transfected Swiss 3T3 fibroblasts expressing TG2 under the control of the tetracycline-regulated inducible promoter, we demonstrate that induction of TG2 not only stimulates an increase in collagen and fibronectin deposition but also an increase in the expression of these proteins. Increased TG2 expression in these fibroblasts led to NFκB activation resulting in the increased expression of TGFβ1. In addition, cells overexpressing TG2 demonstrated an increase in biologically active TGFβ1 in the extracellular environment. A specific-site directed inhibitor of TG abolished the NF-κB and TGFβ1 activation, and the subsequent elevation in the synthesis and deposition of ECM proteins confirming that this process depends on the induction of transglutaminase activity. Treatment of TG2 induced fibroblasts with nontoxic doses of NO donor S-nitroso-N-acetylpenicillamine (SNAP) resulted in decreased TG2 activity and apprehension of the inactive enzyme on the cell surface. This was paralleled by a reduction in activation of NF-κB and TGFβ1 production with a subsequent decrease in collagen expression and deposition. These findings support a role for NO in the regulation of TG2 function in the extracellular environment.

Tissue transglutaminase (TG2) is a member of a family of enzymes that in mammals mediate the Ca2+-dependent formation of e-(γ-glutamyl)-lysine protein cross-links or incorporation of polyamines into proteins to form (γ-glutamyl)-polyamine bonds (1). Previously recognized as an intracellular enzyme, TG2 is now recognized to have a substantial role in the extracellular environment where its functions in cell adhesion and matrix stabilization are not only important in normal wound healing but can also be pathological in diseases such as tissue fibrosis, cancer, coeliac disease and neurodegeneration (1-3).

Export of TG2 to the extracellular space by a yet-unidentified non-classical secretory pathway results in membrane association of the enzyme and its subsequent deposition to the extracellular environment (4,5). This process is reported to be dependent on an intact N-terminal β-sandwich domain, the active-site cysteine (C277) and interaction with integrins (6-8). Via its tight association with fibronectin and independent of its transamidating activity, TG2 can also act as a novel cell adhesion protein to orchestrate both integrin-dependent and RGD-independent cell adhesion and migration (8-10). TG2 can also act as a modulator of the ECM, increasing both its deposition and accumulation by cross-linking of ECM proteins or indirectly via its ability to contribute to the release of the pro-inflammatory and pro-fibrotic cytokine TGFβ1 from its matrix stores (7,9-13). TG2 contributes to the targeting of large latent TGFβ complex by incorporating latent TGFβ binding protein-1 (LTBP-1) into the matrix from which the active TGFβ1 may be released subsequently by proteolysis (13,14,15). TG2
also has a response element for TGFβ1 on its promoter such that activation of TGFβ1 will also result in increased synthesis of TG2 thus propagating the fibrotic cycle (18). Hence, action of TGF-β on cells must be tightly regulated so that the wound healing process does not develop into a fibrotic response with the formation of scarring. Another key player in this cycle of events is NF-κB which is a key regulator of inflammatory gene transcription and is implicated in the induction of TGFβ in monocytes and fibrosarcoma cells (19,20). NF-κB exists in the cytoplasm as a homodimer or heterodimer of the Rel family proteins of p50, p52, c-Rel, RelB, and RelA (p65) which interact with the inhibitory proteins, IκBα, and kept quiescent in the cytoplasm. In response to a stimulus, IκBα undergo proteolytic degradation releasing NF-κB to translocate to the nucleus in a form competent to activate gene transcription (21). Recent evidence indicates that upregulation of TG2 in cancer cells and atherosclerotic smooth muscle cell cultures may promote constitutive NF-κB activation suggesting TG2 as a potential upstream effector in the induction of TGF-β through the NF-κB pathway (22,23).

Nitric oxide (NO), produced during inflammatory events and wound healing nitrosylates free cysteines and forms nitrosothiol adducts that alter the biological activity of proteins (24). TG2 with 18 free cysteines including the critical active site C277 undergoes reversible inhibition of transamidating activity by NO nitrosylation (25,26). NO inhibition of TG2 bioactivity has been reported in mesangial and endothelial cells as well as in chondrocytes (27-29).

Continuous activation of TGFβ-1 leads to excessive deposition of TG2 cross-linked matrix proteins into the ECM which gives rise to fibrotic conditions (30-32). We propose that interaction of NO with TG2 may be important in regulating both its direct effects on matrix crosslinking and in addition, the enzyme’s ability to regulate activation of TGFβ-1 thus forming a key control point in the molecular mechanisms of fibrogenesis and tissue scarring. To investigate this, we have used TG2 transfected Swiss 3T3 fibroblasts expressing TG2 under the control of the tetracycline-regulated inducible promoter as a cell model (12) and a known NO donor S-nitroso-N-acetylpenicillamine (SNAP). We demonstrate, for the first time that TG2 induction stimulates NF-κB activity which is paralleled by an increase in the expression and bioactivation of TGFβ1, with a subsequent elevation in the synthesis and deposition of collagen I, III, and IV and fibronectin into the ECM in a transglutaminase activity dependent manner. In addition, we present new data strongly suggesting that inhibition of TG2 activity by nitrosylation can change the cellular distribution of the enzyme leading to an observed decrease in expression and deposition of matrix proteins and the activity of TGFβ1 and NFκB.

**EXPERIMENTAL PROCEDURES**

**Reagents and antibodies**—

Cell culture medium, growth supplements, and other chemicals of cell culture grade were purchased from Sigma-Aldrich (UK). S-nitroso-N-acetyl penicillamine (SNAP), FN synthetic peptides GRGDTP and GRADSP were from Calbiochem. Rabbit polyclonal antibody against proteins containing nitrotyrosine and human fibronectin were purchased from Abcam and Sigma-Aldrich (UK), respectively. Mouse monoclonal antibodies against NFκβ subunits p50 and p65, IκBα and NFκB inhibitor SN50, were from Santa-Cruz Biotechnology. Human plasma fibronectin and TGFβ1 E{sub max} Immunoassay System Kit, were obtained from Sigma-Aldrich and Promega, respectively (UK). Purified guinea pig liver TG2 (gpl TG2) was purified according to Leblanc et al., (33) and TG2 inhibitor 1,3-dimethyl-2[(oxopropyl)thio]imidazolium derivative (7) R283 was synthesized at Aston University.

**Determination of TG2 antigen and activity**—

Swiss 3T3 cells transfected with human TG2 gene under the control of tetracycline-off promoter were cultured in conditioned DMEM containing 10% fetal calf serum (FCS), 2 mM glutamine, 2 μg/ml tetracycline, 100 U/ml penicillin, 100 μg/ml streptomycin, 400 μg/ml G418, 250 μg/ml xanthine, 10 μg/ml mycophenolic acid and 1xHAT Media Supplement- Hybri-Max (Sigma-Aldrich, UK) as described previously (12). Cells were detached using 0.25% (w/v) trypsin in 5 mM EDTA, and seeded on tissue culture plastic at 5 x 10^4 cell/cm² density overnight in the conditioned media containing 10% FCS. Cell monolayers were washed twice and cells were incubated in the conditioned media containing 2.5% FCS without G418 for 24, 48, or 72 hours. To induce TG2 expression, tetracycline was...
removed from the culture medium. At indicated time points cells were washed and dislodged from their substratum by cell dissociation buffer (PBS, pH 7.4, with 2 mM EDTA) leaving the cell-assembled matrix behind. Plates were washed once with cell dissociation buffer containing 0.1% (w/v) deoxycholate for 5 mins to get rid of remaining intact cells and deoxycholate-soluble cell matrix/debris. The remaining ECM layer was washed three times with PBS, pH 7.4, and solubilized by addition of 2X strength Laemmli gel loading buffer (Sigma-Aldrich, UK) to obtain the ECM fraction. Whole cell lysates (WCL) were obtained by direct solubilization and extraction of proteins from cell monolayers in 50 µl of solubilization buffer (1% (v/v) Nonidet, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM benzamidine, 1 mM NaF, 1 mM Na3VO4, 0.1 mM PMSF and 1% (v/v) protease inhibitor cocktail) as described before (10).

For detection of cell surface TG2 antigen via flow cytometry, detached cells were resuspended in DMEM containing 3 μg anti-TG2 monoclonal antibody Cub7402 (Lab Vision) followed by an incubation with secondary FITC-labeled mouse IgG (3 μg/ml) for 1h at 4°C as previously described (7). The background gate was set with samples incubated with isotype control mouse IgG1 antibody.

TG2 activity was measured in ECM/WCL fractions and cell monolayer of induced and non-induced fibroblast incubated with 0.25 mM fluorescein cadaverin for 72 hours. TG2 mediated fluorescein cadaverin incorporation in ECM/WCL fractions was detected using mouse anti-fluorescein antibody (Roche) by Western Blots, while analysis of incorporation in cell monolayers was performed on cells seeded into permanox 8-well chamber slides by methanol fixation, mounting (Vector Laboratories, UK) and laser confocal microscopy, Zeiss LSM510, using Zeiss LSM Image Browser as described previously (10). ECM and WCL fractions were also screened for expression of FN by immunoblotting with rabbit anti-FN antibody (Sigma) as described previously (12).

**Analysis of Nitrosylated cellular proteins and TG2—**

TG2 induced and non-induced cells seeded on permanox 8-well chamber slides at 5 x 10^4 cell/cm^2 density overnight were treated with 50, 150, 300 μM of NO donor SNAP in low serum conditions for 72 hours as described above. Cells were fixed in 3.7% (w/v) paraformaldehyde in PBS and permeabilized in 0.1% (v/v) Triton X-100 in PBS. For detection of nitrosylated tyrosine containing cellular proteins, cells were blocked with 3% (w/v) bovine serum albumin in PBS (blocking buffer) then incubated with rabbit polyclonal anti-nitrotyrosine antibody (1:100) in blocking buffer, followed by anti-rabbit-FITC diluted 1:200 in the blocking buffer. Slides were mounted and examined by confocal microscopy as previously described (10).

For analysis of nitrotyrosylated TG2, induced cells treated with SNAP were lysed and extracted proteins were pre-cleared in protein G-Sepharose bead slurry with mouse IgG1 antibody (0.5 μg) as described before (10). Pre-cleared cell lysates were incubated with 0.5 μg of anti-TG2 monoclonal antibody Cub7402 for 90 mins at 4ºC, followed by the precipitation of immune complexes with 50 µl of protein A-Sepharose bead slurry for 2 hours at 4ºC. Samples were extracted in Laemmli sample buffer, resolved by 8% SDS gel electrophoresis, transferred to nitrocellulose membrane and immunoprobed with rabbit polyclonal anti-nitrotyrosine antibody.

Nitrosylation of guinea pig liver (gpl) TG2 with S-nitrosocysteine was performed as described by Lai et al., (26). Swiss 3T3 wt cells were seeded on FN matrices enriched by TG2 or nitrosylated TG2 in the presence or absence of G-SPAD or G-SPDP peptides and cell adhesion assays were performed and attachment and spreading were quantified as previously described (10).

**Measurement of collagen deposition by radiolabelling—**

Swiss 3T3 cells transfected with TG2 seeded on the 24 well cultured overnight in the presence of tetracycline were washed and cultured in conditioned medium containing 3%FBS, in the presence or absence of 50-300µM SNAP and 5 μCi/ml of [5-3H] proline (Amersham, UK). After a 48hr-incubation, cell assembled ECM was stripped off cells as described above. Cellular fractions composed of detached cells were solubilized in 0.1% (w/v) deoxycholate for 5 minutes and treated with 200μg/ml of Proteinase K for 30 minutes at 37°C. ECM fractions were extracted via a triple digestion (100 µl/well) with 0.25% (w/v) trypsin, 200μg/ml proteinase K (Sigma-Aldrich, UK) and 0.8mg/ml of collagenase (Sigma-Aldrich, UK), respectively. Proteinase K was deactivated and remaining matrix was scraped into 4% (w/v) SDS which
was combined with triple digest to obtain the full ECM fraction. Radioactive label was analyzed by mixing 100 μl of each fraction with 1 mL of Ultima Gold scintillation liquid and counted in a Packard Instruments Tri-carb 300 scintillation counter for 5 minutes. Results were normalized against protein contents measured in cellular fractions.

Quantitative real-time RT-PCR—
Total RNA was isolated using Trizol reagent (Invitrogen) and reverse transcription was carried out using 200 ng of total RNA at 37°C for 90 minutes using Sensiscript RT Kit (Qiagen) according to the manufacturer's instructions. For detection and quantification of the collagen and TGFβ1 mRNA levels the iCycler iQ Real-Time PCR Detection System (Bio-Rad) was used. Real-time PCR reactions were performed with QuantiFast SYBR Green PCR Kit (Qiagen) according to manufacturer's protocol. Each 25 μl reaction mixture consisted of 500 ng of cDNA, 12.5 μl of SYBR Green PCR Master Mix (Qiagen), and 1 μM designed sense and anti-sense primers (Table 1). The specificity of the reaction products was confirmed by melt curve analysis and by visualization of a single band of correct size using 2 % agarose gel electrophoresis. Each sample was tested in triplicate and the fold change in mRNA levels was calculated by using standard curve method against 18 S rRNA in order to correct differences in both RNA quality and quantity.

Electrophoretic Mobility Shift Assay—
After indicated treatments, nuclear and cytoplasmic proteins were isolated and analyzed for NFκB binding activity by incubating equivalent protein amounts (5 μg) for 30 min at room temperature with 3 pmol of biotin labeled double-stranded oligomer probe, as described before (Caccamo et.al., 2005). The probe used in electrophoretic mobility shift assay (EMSA) was forward 5’- ACAATCAGTTGAGGGACTTTCCCAGGC AAC-3’ and reverse 5’- TTGCCTGGGAAAGTCCCTCAACTGATTG T-3’ which was labeled using Biotin 3’ End DNA Labeling kit (Pierce) according to manufacturer’s instructions.

A competition and supershift assay was carried out by incubating nuclear extracts with unlabelled probe (100X) and anti-p65/p50, respectively, in the binding buffer at room temperature for 45 min (34). Bound complexes were separated onto 8-15% nondenaturing two-step polyacrylamide gels, transferred onto nylon membrane, and detected by LightShift Chemiluminescent EMSA Kit (Pierce) according to manufacturer's protocol.

Statistical Analysis—
Differences between data sets were determined by the Mann–Whitney or Student’s t-test using Minitab package. The difference between two data sets was taken significant when p value was <0.05.

RESULTS
Induction of TG2 expression and activity increases the synthesis and deposition of matrix proteins—
In agreement with our previous report (12) TG2 expression was induced upon removal of tetracycline from the culture medium of transfected Swiss 3T3 cells for 72 hours which was evident in whole cell lysates. Following induction increased TG2 protein was deposited into the cell-assembled ECM laid down by these cells (Fig. 1A). The treatment of cells with the site directed TG inhibitor R283 (500 µM) on a daily basis from the time of induction did not affect the intracellular and extracellular distribution of TG2 antigen. However, R283 did block the TG2-mediated fluorescein-cadaverine incorporation into total cellular proteins including the deoxycholate insoluble matrix proteins (Fig. 1B). R283 is a cell permeable, site directed irreversible inhibitor of TG that binds to the active site cysteine residue of the enzyme leading to acetylation of the thiol group with subsequent release of the complementary thione (35,36). Analysis of the TG mediated crosslink ε-(γ-glutamyl) lysine in whole cell extracts by cation exchange chromatography demonstrated that a two fold increase in the cross link levels of the TG2 induced fibroblasts compared to non-induced which was reduced to undetectable levels in the induced cells treated with the R283 (Fig. 1C).

Recent evidence suggests that the amount of extracellular matrix deposited is directly proportional with the expression levels of TG2 in tubular epithelial cells in an in vitro kidney scarring model (37). To further elucidate whether increased matrix deposition is dependent or independent of TG2 activity, synthesis and deposition of matrix proteins was
investigated. Overexpression of TG2 caused a two fold increase (p<0.05) in the synthesis of FN protein as observed in whole cell lysates but Induced fibroblasts cultured in the presence of TG2 inhibitor R283 failed to show such an increase in FN levels (Fig. 2A). When cell deposited FN matrix was isolated and separated in reducing SDS-PAGE conditions, large molecular weight FN polymers unable to enter the stacking gel were present only in induced cells for TG2. The high-molecular-weight polymers were not visible in FN matrices laid down by non-induced and R283 treated induced fibroblasts (Fig. 2A). To establish the total effect of TG2 induction on collagen synthesis and deposition, cell cultures were labeled with [5-3H] proline and the amount of the radiolabel and deposition, cell cultures were labeled with 

Determination of TGFβ1 mRNA levels by quantitative real-time PCR at time points 24, 48, and 72 hours demonstrated a 40%-50% increase in TGFβ1 expression (p<0.05) in TG2 induced fibroblasts compared with the non-induced cells (Fig. 3A). To investigate whether TG2 induction affected active and total TGF β1 levels in a transamidating activity dependent manner, a sandwich ELISA assay was performed on media collected from induced, non-induced cells for TG2 and induced cells treated with R283 for 48 and 72 hours (Fig. 3B). In comparison to the non-induced control, induced cells showed a significant three fold increase (p<0.05) in active TGFβ1 levels, which was brought down to control levels by R283 treatment. In agreement with the real-time PCR results, total TGFβ1 levels measured in these media samples after acid hydrolysis mirrored the trend observed for the active TGFβ1 levels.

Given the reported involvement of TG2 in NFκB activation (22,38,39) and the ability of NFκB to regulate TGFβ1 expression (19,20), we reasoned that increase in TGFβ1 mRNA levels might be due to upregulation of NFκB activity by a transamidation mechanism. Induction of TG2 in fibroblasts led to activation of the NF-κB pathway as demonstrated by EMSA (Fig.3C). DNA binding activity of NF-κB represented by a large complex of slower mobility was increased in nuclear extracts of induced cells in comparison with non-induced cultures, where levels of active NF-κB complexes were found to be five-fold lower (p<0.05). Supershift experiments with monoclonal antibodies could identify NF-κB p50 and p65 subunits within the large complex of slower mobility (Supplemental Fig.1A). The treatment of induced cells with the TG2 site directed inhibitor R283 resulted in a significant reduction (p<0.05) in the activation of NF-κB. In order to investigate whether TG2 induced NF-κB activation required degradation of IκBα, we assayed the cytoplasmic extracts for IκBα by Western blot analysis (Fig.3D). The overexpression of TG2 in fibroblasts led to a significant 4 fold reduction (p<0.05) in IκBα protein levels compared to non-induced cells, which was reversed when the cells were cultured in the presence of R283, indicating that TG2-induced NFκ-B activation is accompanied by IκBα degradation. To determine the effect of

Increased TG2 expression increases TGFβ1 synthesis and activation in a NFκB dependent manner—Given that regulation of TG2 expression is thought to be important for controlling matrix storage and activation of latent TGFβ1 complexes (15), we next explored the possibility that TG2, in addition to its ability to activate latent TGFβ1 complexes, may also be involved in upregulation of TGFβ1 expression (22)
TG2 mediated NF-κB activation on TGFβ1 regulation, we also cultured TG2 induced cells in the presence of a specific NF-κB inhibitor, SN50, and measured the levels of active and total TGFβ1 in media. The treatment of TG2 induced cells with SN50 significantly reduced the active and total TGFβ1 levels (Fig. 3B) (p<0.05) while no apparent change was observed in that of control non-induced cells (data not shown), suggesting that TG2 mediated activation of NF-κB increases TGFβ1 synthesis and activation.

Nitric oxide is an important regulator of TG2 activity—
Treatment of purified guinea pig liver enzyme TG2 with NO carrier revealed that TG2 can be polynitrosylated resulting in the inhibition of the enzymes transamidating activity (26). To establish the effect of nitrosylation on the cell associated TG2, Swiss 3T3 induced to express TG2 were treated with the NO donor SNAP from the time of their induction for 72 hours. SNAP was chosen as the NO donor in this study due to its sustained release of NO with a half-life of 36.6±4. Other NO donors such as NONOates were not preferred because of their active amine groups which could potentially interfere with TG2 activity(40). LDH cytotoxicity and MTT cell proliferation assays showed 300μM SNAP as the maximum tolerated concentration for induced cells (Supplemental Fig. 1B,C). Griess assay performed on cell supernatants of SNAP treated fibroblasts demonstrated that the treatment increased NO production in a concentration dependent manner for 72 hours (Supplemental Fig. 1D). In addition intense immunostaining for nitrotyrosine residues was seen in induced cells with the increasing SNAP concentration (Fig. 4A). To determine the putative nitrosylation of TG2 by SNAP, TG2 was immunoprecipitated from induced cells cultured in the presence or absence of increasing concentrations of SNAP. Immunoprecipitation of TG2 followed by immunoblotting for nitrotyrosine revealed that TG2 was indeed nitrosylated as the result of SNAP treatment (Fig. 4B). When whole cell lysates extracted from induced cells grown with SNAP was analyzed by Western Blotting for TG2 antigen, no significant change was observed (p>0.05). However, increasing concentrations of SNAP progressively reduced the deposition of TG2 significantly (p<0.05) into the cell assembled ECM (Fig. 4C). Treatment of induced cells with 50 μM and 150 μM SNAP led to a 3 and 6 fold decrease in TG2 antigen levels, respectively, while 300 μM SNAP strongly suppressed the enzyme’s deposition.

In view of these findings, we further investigated whether the reduction of TG2 in ECM was as a result of its reduced export to the cell surface and matrix due to loss of the enzymatic activity followed by SNAP treatment. Detection of cell surface associated TG2 by immunoprobing live non-permeabilized cells followed by flow cytometry analysis demonstrated that SNAP significantly concentrated the enzyme on the cell surface, up to 30% in a dose dependent manner (Fig. 4D).

To investigate the effect of NO on the TG2 activity, induced and non-induced cells were cultured with the fluorescently labeled primary amine substrate cadaverine in the presence of increasing concentrations of SNAP (Fig.5A and B). Induced cells exhibited a strong fibrillar staining pattern for fluorescein-cadaverin incorporation which was reduced to a weakened punctuated pattern by increasing concentrations of NO donor (Fig.5A). In order to quantify the decrease in TG2 activity, cell lysates were collected and subjected to Western Blotting and ε-(γ-glutamyl) lysine crosslink analysis. Immunoprobing of Western blot with anti-fluorescein antibody indicated that SNAP treatment led to 20-60% decrease in the signal level of large molecular weight proteins in WCL and ECM fractions compared to control induced cells (Fig. 5B). The ε-(γ-glutamyl) lysine crosslink analysis confirmed the decrease in TG2 activity with increasing concentrations of NO donor (Fig. 5C).

To verify that NO downregulates TG2-induced collagen biosynthesis, we analyzed the mRNA levels of α1 chain of collagen III, α1 and α2 chains of collagen I and collagen IV. In induced fibroblasts treated with increasing concentrations of SNAP, analysis by real-time PCR at different time points revealed treatment with SNAP led to a significant reduction (p<0.05) in collagen mRNA levels even at low concentrations. At all concentrations the effect of SNAP on collagen 3 α1 chain mRNA levels was a significant 40% decrease (p<0.05) from control levels recorded for TG2 induced fibroblasts (Fig. 6C). However, there is an observed trend in which treatment with increasing concentrations of SNAP decreases expression levels of the collagen I and collagen 4 in a decremental manner. For example, SNAP led to a steady reduction in
mRNA levels of α1 and α2 chains of collagen 1 from 50% to 10% of control levels (p<0.05) (Fig. 6D). A significant 40% to 30% decrease (p<0.05) in collagen type 4 α1/α2 chain mRNA expression was recorded with the increasing doses of SNAP treatment (Fig. 6E).

NO downregulates TG2 mediated increase inTGFβ1 synthesis through NFκB deactivation—

Because SNAP treatment led to decreased collagen synthesis and deposition in fibroblasts induced for TG2, we examined the effect of SNAP on the NFκB dependent regulation of TGFβ1 in these cells. The real-time PCR results showed significant expression of TGFβ1 in TG2 induced cells which was reduced to levels recorded for non-induced cells 24, 48 and 72 hours after SNAP treatment (Fig. 7A). TGFβ1 mRNA was maximaly reduced by 70% (p<0.05) by treatment with 300 μM SNAP, while 50 and 150 μM SNAP led to a significant 50% and 60% decrease at all time points. A similar decline was observed for the levels of biologically active and total TGFβ1 in the media of induced cells due to treatment with SNAP (Fig. 7B). Incubation of induced cells with SNAP for 48 and 72 hours significantly reduced active and total TGFβ1 to 30%, 50% and 60% of control value (without treatment) at 50, 150, and 300 μM SNAP, respectively.

We next examined the impact of increasing amounts of NO on the activation of NFκB in TG2 induced cells. The activation of NFκB in induced cells was suppressed by the addition of increasing concentrations of SNAP (Fig. 7C). At 150 and 300 μM SNAP, the DNA binding activity of NFκB was significantly reduced to 60% and 30% of control NFκB activity for induced cells without SNAP treatment. To determine whether IkBα degradation was preceded by NFκB activation, we examined the protein levels of IkBα in cytosolic fractions by Western Blot analysis. As shown in Fig. 7C, IkBα degradation was evident only for induced cells and induced cells treated with the lower 50 μM concentration of SNAP promoting the nuclear export of NFκxB, parallaeling the higher activation values for NFκB DNA binding in these samples when measured by the EMSA.

DISCUSSION

We demonstrated that elevation of endogenous TG2 levels in tubular epithelial cells (TEC) or the exogenous addition of TG2 to dermal fibroblasts increased the deposition of ECM proteins such as collagen and fibronectin, which was accompanied by crosslinking of the established matrix and reduced matrix turnover rate (11,41). Animal models of kidney scarring have since confirmed these in vitro findings. For example in a subtotal nephrectomy scarring model the application of site directed TG inhibitors reduced the deposition of FN, collagen 1, collagen 3, and collagen 4 ECM proteins in the scarred kidney (42). While in a unilateral ureteral obstruction model of kidney scarring using TG2 knockout animals the loss of TG2 led to the accumulation of less fibrillar collagen, decreased active TGFβ1 and reduced cell infiltration (31).

By utilizing a well characterized TG2 transfected Swiss 3T3 fibroblasts model under the control of the tetracycline-regulated inducible promoter (12), modulation of the expression and activity of the enzyme could be achieved in a controlled manner to give a TG2 activity of around 30 U/mg protein which is comparable to that found in the fibrotic kidney, indicating that TG2 expressed by these cells is relevant to in vivo conditions (12,37,43). Control experiments with wild type Swiss 3T3 cells showed that presence of tetracycline in the culture medium did not have any non specific effects on TG2 and TGFβ1 expression levels (Supplemental Fig.1E) Induction of TG2 in these cells led to a corresponding increase in e-(γ-glutamyl)lysine cross-link levels which could be reduced to undetectable levels when cells were incubated with the specific site-directed irreversible inhibitor of transglutaminase, R283. Importantly, raising the levels of TG2 resulted in translocation of enzyme from the cytoplasmic pool to the extracellular space by a process independent of transamidating activity as R283 treatment did not affect this distribution. In agreement with previous reports on animal models of kidney scarring our data demonstrates that upregulation of TG2 expression and activity causes an increase in the levels of cellular and total ECM collagen, while inhibiting transglutaminase activity lowers collagen biosynthesis (31,42). The increase we observed in collagen deposition seems to be directly related to the increase in the expression of α1/α2 chains of collagen type 1 and 4 and α1 chain of collagen type 3 mRNA. However, the effects of increased TG2 activity on the transcription of collagen in TECs at first glance appear contradictory. While glucose induced TG2
overexpression in TECs and increased collagen deposition in an mRNA dependent manner (11), stable transfection of the same cells with TG2 also yielded collagen accumulation but this was not RNA dependent (37). These paradoxical effects of TG2 on collagen biosynthesis are likely due to the other effects of glucose on gene expression. In addition, unlike TG2 transfected TECs, TG2 induced 3T3 fibroblasts showed increased active TGFβ1 accompanied with an increased TGFβ1 mRNA and total protein that could be reduced by transglutaminase inhibition using the site directed inhibitor R283. The mechanism of TG2 mediated latent TGFβ activation depends on the cross-linking of LTBP-1 to the ECM resulting in the covalent anchorage of latent TGFβ to the ECM in close vicinity of the activator proteases (13,44). Previously we demonstrated that the same TG2 transfected Swiss 3T3 fibroblasts used in this study when induced for TG2 expression showed an increased rate of LTBP-1 deposition in the matrix coupled by an increased pool of high molecular weight FN polymers (15). However, TECs cells are deficient in the synthesis of LTBP-1 clearly affecting the action of TG2 in TGFβ activation (45).

In transfected fibroblasts induced to express TG2, NFκB DNA binding activity was increased in nuclear fractions, however, the addition of the transglutaminase inhibitor R283 into the culture medium blocked the observed impact of TG2, suggesting that the cross-linking activity of TG2 is responsible for the NFκB activation as recently found (22,23). Interestingly NFκB activation paralleled the IκBα degradation, rather than the polymerization of IκBα. This comes in agreement with the findings that silencing of TG2 in breast and ovarian cancer cells or inhibition of TG2 activity by enzyme inhibitors resulted in a dramatic increase in cytosolic levels of IκBα accompanied by inactivation of NFκB (38,39). In previous studies, silencing of the RelA (p65) unit of NFκB resulted in a direct inhibition of TGFβ1 expression placing NFκB as a central mediator in the induction of TGFβ1 in fibrosarcomas and monocytes (19,20). Of relevance, treatment of TG2 induced cells with NFκB inhibitor blocked active and total TGFβ1 protein suggesting that induction of NFκB may be the key component in the molecular mechanism underlying the stimulatory effect of TG2 on TGFβ1 synthesis.

A further objective in undertaking this study was to investigate whether modulation of cellular TG2 activity by NO would be able to influence the effects of TG2 on NFκB dependent TGFβ1 driven synthesis and deposition of ECM proteins. It has recently been demonstrated that in small artery remodeling in hypertensive induced TG2 wt and TG2 ko mice dosed with the NO inhibitor L-NAME, the lumen diameter of mesenteric arteries was substantially increased in the wt mice while inward remodeling was absent in TG2 ko mice (46). In this report, we demonstrate that TG2 can be nitrosylated at the cellular level when Swiss 3T3 fibroblasts expressing TG2 were treated with a known NO donor (SNAP) at non-toxic concentrations. In transfected cells induced to express catalytically active TG2, SNAP treatment not only decreased the enzyme activity but also reduced the deposition of TG2 into the ECM with a subsequent increase of the enzyme at the cell surface. Together with S-nitrosylation, tyrosine nitrosylation of TG2 might be responsible for the inactivation and distribution of the enzyme since previously we showed that the active site cysteine (C277) and tyrosine (Y274) residues play an important role in the activity and deposition of the enzyme into the extracellular environment. As expected SNAP treatment reduced the TG2 mediated increase in FN and collagen biosynthesis and deposition into the ECM in a comparable manner to R283. Importantly, increasing concentrations of SNAP resulted in a decline in active and total TGFβ1 protein that was mRNA dependent. The decline in TGFβ1 levels was in line with reduced NFκB activation. The possibility that NO oxide exerts these effects independently from the negative regulation of transglutaminase activity was unlikely since treatment of non-induced cells with SNAP did not cause any significant changes on these parameters (data not shown). Our results therefore point to a key role for NO in regulating TG2 mediated NFκB dependent TGFβ1 activation associated with increased ECM protein synthesis and deposition. TG2 appears to synergise with TGFβ1 regarding the ECM synthesis and tissue remodeling in an activity dependent manner (1,47-49). A failure of the normal wound healing response to terminate, which is generally associated by a continuing insult, gives rise to excessive scarring due to excessive production and contraction of connective tissue. This in turn leads to a pathological healing processes such as fibrosis in which active TGFβ1 and active TG2 are known to take part (16,30,50,51). Increasing evidence
suggests that the initiation of the fibrotic response is associated with a progressive decrease in NO synthesis and NO availability (27,52-56). It may therefore be plausible that NO contributes to both the fine tuning of the matrix deposition of transglutaminase and its matrix activity thus regulating TGFβ1 bioavailability by rendering the enzyme in an inactive confirmation but still enabling it to serve as a novel cell adhesion protein (9,10). Important to our hypothesis is the finding that nitrosylation of TG2 has no effect on the binding efficiency of the enzyme to FN (data not shown) to form a FN-bound nitrosylated TG2 (FN-nsTG) complex which could mediate the RGD-independent cell adhesion (Fig.8) comparable to FN bound TG2 complex (FN-TG).

In conclusion our results shown in this paper using fibroblasts allow us to propose a novel regulatory pathway for TG2 activity. In this pathway NO neutralizes the active form of the enzyme thereby controlling the production and activation of TGFβ1 through an NFκB dependent pathway thus helping to prevent excessive connective tissue accumulation and facilitating the enzyme’s functions as a novel cell adhesion protein which also has an important role in the wound healing process (2).

REFERENCES


FOOTNOTES
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FIGURE LEGENDS

Figure 1. Localization and activity of TG2 in cultures of transfected Swiss 3T3 fibroblasts in the presence of R283.
Swiss 3T3 fibroblasts induced to overexpress TG2 (Ind) was treated with 500 μM R283 from the time of induction. After 72hours, cells expressing endogenous levels of TG2 (NI), and Ind fibroblasts ± R283 were analyzed for TG2 antigen (A) and TG2 activity by the incorporation of FITC-labeled cadaverin by Western Blotting (B) in whole cell lysates (WCL) and extracellular matrix (ECM). High molecular weight multimer proteins are present at the top of the stacking gel (SG) as indicated by molecular weight markers (right, kDa). (C) Measurement of ε-(γ-glutamyl)lysine in NI, Ind, and Ind fibroblasts treated with R283 (Ind R283) via cation exchange chromatography. ε-(γ-glutamyl)lysine values (picomole) were normalized against the mg of protein in each sample. Blots represent one typical experiment and values are expressed as mean ± S.D. from at least three independent experiments.

Figure 2. Effect of increased TG2 expression on the synthesis and deposition of fibronectin and collagen.
(A) Cells induced for 72 hours (Ind) to overexpress TG2 with or without 500μM R283 and non-induced cells (NI) were analyzed for the levels of fibronectin (FN) antigen in whole cell lysates (WCL) and extracellular matrix fractions (ECM) by Western Blotting as described in Experimental Procedures. SG indicates the stacking gel and molecular weights (kDa) are shown to the right of blots. Blots represent one typical experiment of five. (B) Cells were cultured for 48 hours in the presence of [2,3-3H]-proline and the collagen synthesis in cellular fractions and deposited collagen in extracellular matrix were measured (Experimental Procedures). Data is expressed as the mean percentage of cpm value measured for Induced cell ± S.D. from at least five independent experiments performed in duplicates. (C-E) Total RNA extracted from noninduced (NI) and induced cells (Ind) at 24, 48, and 72 hour-time points were subjected to real-time PCR using primers designed for collagen type 3 α1 (C) chain, collagen type 1 (D), and collagen type 4 α1 and α2 chains (E). Collagen mRNA levels were normalized to 18sRNA mRNA (means ± S.E. of the mean for three independent experiments).

Figure 3. Role of TG2 in the activation of TGFβ1 and NFκB
(A) TGFβ1 mRNA was quantitated by real-time RT-PCR and normalized to 18sRNA mRNA (means ± S.E. of the mean for three independent experiments) in non-induced (NI) and induced (Ind) cells cultured for 48 and 72 hours. (B) Noninduced and induced cells treated with R283 or NFκB inhibitor SN50 were cultured and biological activity of TGFβ1 in the sample supernatants was measured using a sandwich ELISA assay as described by the manufacturer at 48 and 72 hours. Following acid activation total TGFβ1 levels were also analyzed. Data represents mean values ± S.D. per sample from at least three separate experiments performed in triplicate. (C) Representative EMSA blots showing the activity levels of NFκB in nuclear extracts of noninduced (NI), induced (Ind) and induced cells treated with R283 and or NFκB inhibitor SN50. Free biotin-labeled (lane1) and
unlabeled NFkB promoter probe in 100 fold excess (lane 5) was added as a control. (D) The protein levels of IkBα was analyzed in whole cell lysates of noninduced, induced± R283 by Western Blotting. All blots are representative of at least three experiments.

**Figure 4. Effect of nitric oxide on the distribution of TG2.**

(A) Representative confocal images of NO donor S-nitroso-N-acetylpenicillamine (SNAP) (50, 150, and 300 μM) treated Induced fibroblasts stained for anti-nitrotyrosine antibody. (B) Lysates of TG2 induced cell samples treated with SNAP at indicated concentrations were immunoprecipitated with anti-TG2 antibody Cub7402 and Western-blotted for anti-nitrotyrosine antibody as described under “Experimental Procedures”. (C) Levels of TG2 antigen in whole cell lysates and ECM fractions in noninduced cells and induced cells treated with increasing concentrations of SNAP were detected by Western Blotting. (D) Cell surface TG2 antigen levels were detected in the cell samples immunoprobbed with anti-TG2 antibody Cub7402 followed by FITC-conjugated secondary antibody using flow cytometry as described (Experimental Procedures). Mouse IgG was used as the isotype control to set the background gate.

**Figure 5. Effect of nitric oxide on the TG2 enzymatic activity.**

TG2-mediated incorporation of fluorescein-cadaverine into the ECM laid down by TG2 was measured in induced cells treated with 50, 150, and 300 μM SNAP. Cells were cultured with 5mM fluorescein-cadaverine for 72 hours, fixed and viewed by confocal microscopy as described under Experimental Procedures. (B) FITC-cadaverin levels were determined in whole cell lysates (WCL) and extracellular fractions of cells by Western Blotting. (C) Data from cation exchange chromatography showing the ε-γ-glutamyl cross-link analysis normalized with the mg protein content in induced cells after SNAP treatment. Blots represent one typical experiment and values are expressed as mean ± S.D. from at least three independent experiments.

**Figure 6. Effect of nitric oxide on TG2 mediated synthesis and deposition of fibronectin and collagen.**

(A) Fibronectin (FN) synthesis in whole cell lysates (WCL) and deposition to extracellular matrix (ECM) was measured by Western Blotting in noninduced and induced cells treated with 50, 150, and 300 μM SNAP. Molecular weight markers (kDa) and stacking gel (SG) are labeled at the right of the blots. (B) Radioactive labeling of cultures with [2,3-3H] proline was undertaken as described under “Experimental Procedures”. Data show radioactivity present in cellular and ECM fractions and was expressed as percentage of cpm values recorded for induced cells (100%), and represent one of five separate experiments performed in duplicate. (C-E) Comparison of collagen type 3(C) collagen type 1 (D), and collagen type 4 α (E) expression levels in induced cells treated with increasing concentrations of SNAP at 24, 48, and 72 hour-time points by real-time quantitative PCR as described in “Experimental Procedures”. Collagen mRNA levels were normalized to 18sRNA mRNA (means ± S.E. of the mean for three independent experiments).

**Figure 7. Role of nitric oxide in TG2 induced activation of TGFβ1 and NFkB**

(A) Expression levels of housekeeping gene 18sRNA and TGFβ1 were measured by quantitative real time PCR in induced cell samples treated with SNAP at 50, 150, and 300 μM concentrations. Data is normalized with 18sRNA and expressed as means ± S.E. of the mean for three independent experiments. (B) Biologically active and total TGFβ1 levels in media collected from induced cell samples treated SNAP were analyzed by sandwich ELISA. Data represents mean values ± S.D. per sample from at least three separate experiments performed in triplicate. (C) Induced cells were treated with indicated concentrations of SNAP and NFkB inhibitor (SN 50) and nuclear extracts were harvested and EMSA was performed using the biotin labeled NFkB probe as described in “Experimental Procedures”. (D) Cell lysates were assessed by Western blotting for IkB levels.

**Figure 8. Nitrosylation of TG2 does not affect its ability to act as an RGD independent protein cell adhesion protein when complexed with FN.**
Cell attachment and cell spreading of Swiss 3T3 wt fibroblasts on FN and TG2 immobilized FN (FN-TG) and nitrosylated TG2 immobilized FN (FN-nsTG) matrices were assessed 20 min after seeding cells that were pre-incubated with increasing concentrations of RGD synthetic peptide (100 and 150μM) as described previously (10). Data are expressed as percentage of control values of cells treated with inactive control RAD peptide, which represents 100% and represent one of at least 3 separate experiments performed in triplicate.
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Abbreviations: Col 1A1, Colagen Type 1 α1 chain; Col 1A2, Colagen Type 1 α2 chain; Col 4A1, Colagen Type 4 α1 chain; Col 4A2, Colagen Type 4 α2 chain; Col 3A1, Colagen Type 3 α1 chain, TGFβ1, Transforming Growth Factor β1; 18sRNA, 18S ribosomal RNA.

Table 1: List of forward and reverse murine primers used for real-time RT PCR.
Figure 1
Figure 2
Figure 3
Figure 3
Figure 4

A

0 µM 50 µM

150 µM 300 µM

B

IgG Cont 1%
Ind 64.8 %

IgG Cont 1%
Ind 50µM 75.8 %

IgG Cont 1%
Ind 150µM 72.8 %

IgG Cont 1%
Ind 300µM 96.1 %

C

Nitrosylated TG2

D

Counts

FITC
Figure 5

A

B

C

Figure 5

SNAP (µM)

0 50 150 300

ε-(γ-glutamyl) lysine (pmol/mg protein)

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8

0 50 150 300

0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8

WCL FITC

ECM FITC

Actin

NI Ind

SG

SNAP µM 50 150 300

50 160 105 75 50

0 µM 50 µM 150 µM 300 µM
Figure 6
Figure 6

(a) and (b) show the expression levels of Col1α1 and Col1α2 mRNA under different SNAP concentrations (0, 50, 150, 300 µM) at 24, 48, and 72 hours. The expression levels are normalized to 18S rRNA levels.

(c) and (d) illustrate the expression levels of Col1α1 and Col1α2 mRNA at different time points (24, 48, 72 hours) for each SNAP concentration (0, 50, 150, 300 µM).

(e) and (f) depict the expression levels of Col4α1 and Col4α2 mRNA under the same conditions as above.
Figure 7
Figure 7

NFκB

Probe

SNAP (µM)  50 150 300

IκBα

SNAP (µM)  50 150 300

Actin

NI  Ind

Figure 7
Figure 8

Cell Attachment

Cell Spreading

FN  FN-TG  FN- nsTG

RAD  RGD 100  RGD 150

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