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Elevated expression of isopeptide bond cross-links contributes to fibrosis in scleroderma and the healing wounds of tight skin mice

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Abstract

Scleroderma is a chronic disease characterized by excessive tissue fibrosis. Recent studies indicate that cultured dermal fibroblasts isolated from patients produce excessive amounts of collagen and other extracellular matrix components. In this study, we investigated the mechanism(s) of abnormal extracellular matrix accumulation in the scleroderma biopsies and the healing wounds of Tsk1/+ mice. Full-thickness excisional wounds were made in Tsk1/+ and wild-type mice and were subsequently harvested at days 7, 10, and 14 postinjury. The levels of profibrotic cytokine, transforming growth factor were elevated in the wounds of Tsk1/+ mice. Interestingly, the levels of matrix metalloproteinase were significantly reduced in the granulation tissue of Tsk1/+ mice in comparison with wild-type. Furthermore, immunohistochemical analysis of the wounds indicated that the levels of γ -glutamyl- ϵ -lysine cross-links were elevated in the granulation tissue of Tsk1/+ mice as well as the fibrotic lesions of scleroderma specimens. Collectively, these findings indicate that elevated collagen synthesis and decreased matrix metalloproteinase levels, in combination with increased isopeptide bond cross-links, contribute to abnormal collagen synthesis and assembly in granulation tissue of Tsk1/+ mice and the fibrotic lesions of scleroderma patients.

Scleroderma is an intricate disorder of connective tissue that initially affects the skin but later also involves internal organs such as the kidneys, lungs, gastrointestinal tract, and cardiovascular system.¹ The key morphological features are (a) structural and functional vascular abnormalities, (b) mononuclear cellular infiltrates that very likely reflect an autoimmune disturbance, and (c) increased deposition of newly synthesized matrix, mainly type I collagen.¹ The morbidity of scleroderma largely depends upon a third facet of the disease—fibrosis. Recent studies have focused on identifying the ways in which different cellular components interact to culminate in the major vascular and fibrotic pathology of scleroderma.^{2,3} Experimental evidence from in vitro and in vivo analyses of cells and tissues from scleroderma patients, as well as from animal models, point to a complex cascade of primary and secondary mediators.^{4,5}

Perhaps the most striking feature of the pathology in scleroderma is the excessive deposition of extracellular matrix (ECM) components, which is the basis for the destruction of normal tissue architecture that leads to tissue and organ dysfunction.^{6,7} Collagen is the predominant component of the ECM and is synthesized and secreted by fibroblasts. It has been demonstrated that cultured dermal fibroblasts from scleroderma patients produce more collagen type I and

III than fibroblasts from healthy individuals.^{8,9} Furthermore, serum from scleroderma patients promotes the growth of high collagen-producing skin fibroblasts clones in vitro.¹⁰ Fibroblast selection could reflect an enhanced response to growth factors, and/or a diminished sensitivity to apoptosis.¹¹ Thus, it is plausible that scleroderma is associated with clonal selection of high collagen-producing fibroblasts.

Examination of scleroderma dermal fibroblasts, using cDNA microarrays, demonstrated that gene expression profiles were consistent with exposure to transforming growth factor-beta 1 (TGF- β 1).¹² Furthermore, increased levels of collagen IV and TIMP3 were observed. It is clear that the expression or regulation of a large number of growth factors, specifically soluble profibrotic mediators such as TGF- β 1, and connective tissue growth factor (CCN2), cytokines (IL-4, IL8, MCP-1), and signaling intermediates are altered in scleroderma.^{13–16} Inflammatory cell recruitment may also be involved in the activation of fibroblasts.¹⁷

Transglutaminases (TG) are calcium-dependent enzymes that catalyze the formation of an isopeptide bond, either within or between polypeptide chains.¹⁸ These γ -glutamyl- ϵ -lysine cross-links are resistant to enzymatic degradation and thereby enhance the stability of tissues. The formation of cross-links appears to be irreversible, because an enzyme with the ability to cleave the cross-links has not been identified. TGs are abundant enzymes that are involved in a number of different physiologic processes.¹⁹ Several ECM proteins such as collagens, fibrillin, fibrinogen,²⁰ osteonectin, and osteopontin²¹ have been identified as substrates for tissue (t)TG. The enzyme contributes to the stabilization of ECM, as shown for calcifying cartilage, basement membranes during matrix assembly, and wound healing.^{20,22}

Among the components of the ECM, a small group of proteins that includes TSP1, TSP2, secreted protein acidic and rich in cysteine, tenascin-C, vitronectin, and osteopontin interact with a variety of cell surface receptors and growth factors. In attempts to gain an understanding of the functional properties of TSP2, TSP2-null mice were generated.²³ Following detailed assessment, these mice were found to exhibit structural and functional abnormalities that encompassed an array of cells and tissues. The phenotype was dominated by abnormalities in connective tissues such as skin, tendon, and ligament.²⁴ It has been reported that TSP2-null dermal fibroblasts have an adhesion defect that was associated with increased levels of matrix metalloproteinase (MMP)-2 in the medium of cultured fibroblasts.²⁴ In addition to degradation of the ECM, which alters the organization as well as the composition of ECM, MMP2 is capable of degrading cell-surface tissue transglutaminase, tTG.²⁵ In vitro studies have confirmed that cell-surface tTG was susceptible to cleavage by MMP2. In deed, it has been documented that tTG activity was reduced in TSP2-null dermal fibroblasts, and as a possible consequence collagen solubility was increased in TSP2-null skins.²⁵

Abnormalities associated with collagen assembly in scleroderma patients have been reported. Expression of lysyl oxidase, which forms lysyl-derived cross-links in collagen, has been documented to be increased in scleroderma biopsies.²⁶ Electron microscopy of fibrotic lesions of scleroderma patients has revealed considerable disorganization of fibrillin bundles.²⁷ Moreover, abnormalities in structural integrity and assembly have been recognized in fibrillin 1-containing microfibrils in the ECM of primary dermal fibroblasts explanted from scleroderma patients.²⁸ Finally, elevated levels of TIMP1 and TIMP2, inhibitors of MMP2, have been observed in scleroderma fibroblasts and patients' sera.²⁹

Tight skin 1/+ mouse is an extensively utilized model of scleroderma-associated dermal fibrosis.^{30,31} In this study, we utilized Tsk1/+ mice to investigate whether increased isopeptide bond cross-links can result in the production of a sclerotic matrix that is resistant to degradation.

MATERIALS AND METHODS

Human skin specimens

Formalin-fixed, paraffin-embedded human skin samples from patients diagnosed clinically and histologically with scleroderma, as well as normal control skin samples, were retrieved from the tissue archives of the University of South Alabama Department of Pathology. The scleroderma samples had been submitted with the clinical suspicion of a connective tissue disorder while the normal control samples consisted of skin removed incidentally at the time of cosmetic surgical procedures for skin laxity. Both scleroderma and control samples were coded with unique identifying numbers to protect patient identity. Because of the nature of the experiments, the study was exempted by the IRB.

Animal models

Tsk1/+ (B6.Cg-Fbn1Tsk1/+ Plidnpa/J) and C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were kept under pathogen free conditions throughout the experiments. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of South Alabama.

Wounds

Animals were anesthetized with isoflurane gas, the dorsal skin was shaved and cleaned with 70% alcohol, and two 6-mm full-thickness excisional wounds were created with the aid of a biopsy punch (Medex Supply, Brooklyn, NY). At days 7, 10, and 14 postinjury animals were euthanized by CO₂ inhalation and the wounds were harvested with a 2-mm surrounding unwounded skin. All mice used in these experiments were approximately 3 months of age and were sex matched. All wounded animals were housed individually, and a total of four mice per genotype per time point were analyzed.

Tissue processing and histology

Uninjured skin and wound specimens were fixed in 10% zinc-formalin buffer (Z-fix, Anatech, Battle Creek, MI), paraffin-embedded, and sectioned at 5 µm thickness, as described previously.²⁵ Sections were subsequently stained with hematoxylin and eosin (H&E) or Masson's trichrome stain according to standard procedures.

Immunohistochemistry

Paraffin-embedded sections were stained with antibodies against MMP2 (Abcam, Cambridge, MA) as described previously.³² Briefly, paraffin-embedded sections were dewaxed with xylene, and rehydrated through a graded concentration of ethanol. Samples were treated with 3% H₂O₂ in methanol to quench endogenous peroxidase. After a series of phosphate buffered saline (PBS) washes, sections were blocked in 1% BSA for 30 minutes, incubated with primary antibodies for 1 hours, washed, and treated with the appropriate biotinylated secondary antibodies. Color development was achieved by incubating the slides with avidin-biotin-horseradish peroxidase complex (ABC-HRP, Vector Laboratories, Burlingame, CA), followed by treatment with 3-3'-diaminobenzidine (Vector Laboratories). All steps were carried out at room temperature. Sections were examined with the aid of an Eclipse 800 light microscope (Nikon, Tokyo, Japan). For morphometric analyses, images were captured with a digital camera and analysis was performed with NIS Elements Software (Nikon Corporation, Melville, NY) as described previously.³³ Briefly, for the histomorphometric analyses of MMP2, a threshold was placed representing the maximum background intensity observed in control (no primary antibody) sections. The values obtained are arbitrary and represent the relative levels and distribution of peroxidase above background for each specific antibody. Analyses of images were carried out independently by two investigators. Similarly, detection of cross-link

formation in paraffin-embedded sections of murine wounds and human specimens were carried out through immunodetection of isopeptide bonds with specific antibodies to ϵ -lysyl γ -glutaminy cross-links (Abcam), as described previously.²⁵ Four sections per sample were analyzed. No sex-related differences were seen in the levels of MMP2 and isopeptide bonds cross-links.

Preparation of wound extracts

For the preparation of wound extracts, a 3-mm biopsy punch was used to excise wound tissue from the center of each excisional wound. Wound tissue ($n = 4$) was homogenized with a polytron homogenizer in 1mL PBS containing 2 mM phenylmethylsulfonyl fluoride and 1 μ g/mL each of aprotinin, leupeptin, and pepstatin A (Sigma Chemical Company, St. Louis, MO) and sonicated for 1 minute on ice. The debris was removed by centrifugation and the protein contents of wound extracts were determined by the BCA assay according to the manufacturer's instructions (Bio-Rad, Hercules, CA).

Determination of active TGF- β 1 protein levels in wounds

Wound tissues ($n = 4$) were prepared as described above and levels of active TGF- β 1 protein in 100- μ L samples were determined using a commercially available enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN). Background levels were subtracted from all measurements.

Determination of MMP2 levels in wounds by zymography

Wound extracts were subjected to SDS-PAGE, under non-reducing conditions, in 7.5% acrylamide gels containing 0.1% gelatin. Protein concentrations were determined as described earlier, and equal amounts of proteins were applied to the gel. After electrophoresis, the gels were washed with gentle shaking for 2 hours at room temperature in 50mMTris-HCl buffer (pH 7.5) containing 100mMNaCl and 2.5% Triton-X 100. Subsequently, the gels were incubated at 37 °C for 18–20 hours, in the same buffer containing 10mM CaCl₂. The gels were then stained with Coomassie blue. Zones of proteolysis appeared as clear bands against a blue background.

Statistical analysis

All results are expressed as mean $\pm$ SD. Statistical significance was evaluated by two-tailed unpaired Student's t -test for comparisons between the means of two groups. A value of $p < 0.05$ was considered to be significant.

RESULTS

Histological analysis of excisional skin wounds

Gross examination of healing wounds revealed that Tsk1/+ mice exhibited delayed healing, as indicated by delayed wound closure and the persistence of scabs (not shown). Examination of H&E-stained days 7, 10, and 14 wound sections revealed prominent differences in the granulation tissue of the mutant mice, in comparison with that in wild-type animals (Figure 1). Specifically, ECM deposition in the wound bed of Tsk1/+ animals was increased in comparison to that of the wild-type wounds.

TGF- β 1 levels in wounds

To determine whether elevated ECM deposition could be attributed to the increased TGF- β 1 levels, we evaluated the level of this cytokine in the extracts of excisional wounds. ELISA analysis indicated that levels of TGF- β 1 protein were elevated in Tsk1/+ wounds at days 7, 10,

and 14 postinjury in comparison to that in wild-type mice (Figure 2). The increase in TGF- β 1 is consistent with the fibrosis in Tsk1/+ mice.

MMP2 levels in excisional wounds

To further investigate alterations in ECM deposition and remodeling in Tsk1/+mice, we examined the deposition of MMP2. We chose to focus on MMP2 because we have previously shown that it is predominant gelatinase secreted by dermal fibroblasts. Immunohistochemical analysis (Figure 3A–F) suggested that the levels of MMP2 were significantly reduced in the granulation tissue of Tsk1/+ mice at all time points examined. Histomorphometric evaluation confirmed the reduced deposition of MMP2 in Tsk1/+ wounds (Figure 3H). Gelatin zymography revealed that both pro- and active MMP2 were reduced (Figure 3G).

Isopeptide bond cross-links in excisional wounds

We have previously demonstrated that tTG is subjected to degradation by the proteolytic activity of MMP2.²⁵ In the same study, we showed that the corresponding levels of isopeptide bond cross-links were significantly decreased in day 10 wounds of TSP2-null mice, which were shown to have increased MMP2.^{24,25} Hence, we evaluated the levels of isopeptide bond cross-links in excisional wounds of Tsk1/+ mice and their wild-type counterparts. Wound sections were stained with antibodies to cross-links, which were found to be elevated in Tsk1/+ mice (Figure 4A–F). Histomorphometric analysis of images confirmed that isopeptide cross-links were elevated in wounds of Tsk1/+ mice (Figure 4G).

Elevated isopeptide bond cross-links in fibrotic lesions of scleroderma specimens

Fibrosis, a major hallmark of scleroderma, has been characterized by increased levels of hydroxylysine aldehyde derived collagen cross-links present in cultured scleroderma fibroblasts.³⁴ We thus examined whether levels of ϵ -lysyl γ -glutamyl cross-links were altered in the scleroderma specimens, which could also attribute to fibrosis. As shown in Figure 5, the levels of isopeptide bond cross-links were elevated in scleroderma specimens (A) in comparison to control samples (B). The observations were confirmed by histomorphometry (Figure 5C).

DISCUSSION

The Tsk1/+ mouse has proved to be a useful experimental animal to study the pathogenesis of fibrosis in scleroderma. In efforts to understand the mechanism of fibrosis in scleroderma, we have utilized a full-thickness excisional wound model to evaluate the parameters of healing in Tsk1/+ mice. Here, we have shown that levels of TGF- β 1 and MMP2 in the granulation tissue of Tsk1/+ mice are significantly increased and reduced, respectively. Furthermore, the concentration of isopeptide bond cross-links, the product of catalysis by tTG, were elevated in dermal wounds of Tsk1/+ mice and fibrotic lesions of scleroderma patients.

We have previously shown that MMP2 was increased in TSP2-null dermal fibroblasts.²⁴ Consistent with our vitro observations, we have observed increased levels of MMP2 in TSP2-null mice during the formation of foreign body granulomas.³² We have also shown that the increase in MMP2 in TSP2-null fibroblasts was associated with reduced tTG levels, presumably through the ability of MMP2 to target the cell surface tTG.²⁵ In addition, collagen solubility was found to be increased in TSP2-null dermis. We thus hypothesize that, in contrast to the findings in TSP2-null mice, stimulation of collagen synthesis by TGF- β 1, together with a combination of reduced MMP2 and increased tTG activity, leads to overproduction of a fibrotic matrix that is difficult to degrade in scleroderma.

tTG is widely distributed in mammalian tissues and contributes to many posttranslational modification events including, but not limited to, amine incorporation, acylation, and cross-linking.¹⁸ tTG participates in collagen fibrillogenesis and can also improve matrix integrity by cross-linking an array of proteins in the extracellular milieu.²⁵ Previous studies in TSP2-null mice indicated that elevated tTG-catalyzed isopeptide bond is responsible for the increase in solubility of dermis.²⁵ Increased tTG expression and activity at sites of neovascularization and invasion of the fibrin matrix, and later in the granulation tissue matrix during healing have been observed.³⁵ tTG appears to be an important participant in the remodeling phase of wound healing, a time-point at which normal healing process relies on maintenance of the original structural integrity of tissue. Interestingly, microfibrils produced by both Tsk1/+ and scleroderma lesional fibroblasts appear unstable and less abundant than those produced by control cells.³⁶ Here, we demonstrate that isopeptide bond cross-links are elevated in excisional dermal wounds of Tsk1/+ mice and fibrotic lesions of scleroderma patients (Figures 4 and 5).

Our data indicate that the level of TGF- β 1 is significantly elevated in the granulation tissue of Tsk1/+ mice. The phenotypic abnormality associated with thickened dermis in Tsk1/+ mice has been linked to increased collagen hydroxyl-proline content and solubility.³⁷ It has been demonstrated that Col1a1 and Col1a2 transcripts are increased in Tsk1/+ mice.³⁸ Furthermore, the activation of the TGF- β 1 signaling pathway has shown to be important in the development of thickened skin. Supporting evidence for these observations is provided by experiments demonstrating that thickened skin phenotype can be ameliorated by crossing the Tsk1/+ mouse with mouse lines that are deficient in TGF- β 1 signaling.³⁹ Crossing Tsk1/+ with mice lacking the TGF- β 1 receptor also appears to abolish the phenotype, while a polymorphism in the TGF- β 1 promoter of Tsk1/+ mice tends to enhance expression of the gene.⁴⁰

In conclusion, we have documented that increased covalent intermolecular cross-links in collagen matrices can culminate in the production of fibrotic matrices in scleroderma and Tsk1/+ granulation tissues. These changes are associated with dysregulation of MMP2 and TGF- β 1 in a manner that favors the production of more highly cross-linked collagen. We speculate that these changes render the skin of Tsk1/+ mice fibrotic. These findings should enable us to develop therapeutics, such as localized gene therapy through reduction of tTG expression via antisense tTG or siRNA, aimed at ameliorating fibrosis.

Glossary

ECM	Extracellular matrix
MMP	Matrix metalloproteinase
TGF-β1	Transforming growth factor-beta 1
Tsk1/+	Tight skin mouse
tTG	Tissue transglutaminase

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Figure 1.

Increased fibrosis in wounds of *Tsk1*^{+/+} mice. Representative sections from days 7, 10, and 14 wounds of *Tsk1*^{+/+} (A–C; G–I) and wild-type (D–F; J–L) mice were stained with H&E (A–F) and Masson's trichrome (G–L). Increased and tightly packed collagen fibers can be observed in *Tsk1*^{+/+} wounds. Scale bar = 50 μ m.

Figure 2.

Elevated levels of active TGF-β1 in Tsk1/+ wounds. TGF-β1 protein levels in days 7, 10, and 14 excisional wounds were quantified. Levels of active TGF-β1 in Tsk1/+ wounds (closed bars) were significantly elevated compared with levels in wild-type wounds (open bars). WT, wild-type; Tsk, Tsk1/+. Experiments were performed in duplicate. Bars indicate the mean $\pm$ SD ($n = 8$); *significantly different from control, $*p \leq 0.05$.

Figure 3.

Decreased expression of MMP2 in Tsk1/+ wounds. Representative sections from Tsk1/+ (A–C) and wild-type (D–F) wounds stained with an anti-MMP2 antibody and visualized with the peroxidase reaction are shown. Decreased deposition of MMP2 is evident in Tsk1/+ wounds (A–C). MMP2 levels were also evaluated by gelatin zymograph, levels of MMP2 are significantly lower in days 7, 10, and 14 wound extracts of Tsk1/+ mice (Figure 3G; panels 1–3, respectively) in comparison with that of days 7, 10, and 14 wound extracts of wild type (Figure 3G; panels 4–6, respectively). Relative levels of MMP2 in days 7, 10, and 14 wounds from wild-type (open bars) and Tsk1/+ (closed bars) mice were measured using metamorphic analysis following immunolocalization with antibodies to MMP2 (H). Arrows denote elevated

MMP2 levels associated with cells and extracellular matrix. Scale bar = 10 μ m. WT, wild-type; Tsk, Tsk1/+. Experiments were performed in triplicate. Scale bars indicate the mean $\pm$ SD (n = 8); *significantly different from control, * $p \leq 0.05$.

Figure 4.

Detection of isopeptide bonds in wounds. Isopeptide bond formation is increased in day 7, 10, and 14 wounds of Tsk1/+ mice (A–C) in comparison with WT tissue (D–F). Wound sections were stained with antibodies against isopeptide bonds, and visualized by the peroxidase reaction. NIS Elements software was used to quantify the staining intensity in wild-type (open bars) and Tsk1/+ (closed bars) wounds. A total of 40 images from four wounds per time point per genotype were analyzed. AU, arbitrary units. WT, wild-type; Tsk, Tsk1/+. Arrows denote elevated isopeptide bond formation associated with cells and extracellular matrix. Scale bar = 10 μ m. Experiments were performed in triplicate. Bars indicate the mean $\pm$ SD ($n = 8$); *significantly different from control, $*p \leq 0.05$.

Figure 5.

Detection of ϵ -lysyl γ -glutaminy cross-links in scleroderma specimens. Isopeptide bond formation is increased in scleroderma specimens (A) in comparison with the control samples (B). Paraffin-embedded scleroderma and control specimens were incubated with antibodies against isopeptide cross-links, and visualized by the peroxidase reaction. NIS Elements software was used to quantify the staining intensity in fibrotic lesions. A total of 40 images from four samples were analyzed. AU, arbitrary units. Arrows denote elevated isopeptide bond formation associated with cells and extracellular matrix. Scale bar = 10 μ m. Scale bars indicate the mean $\pm$ SD ($n = 2$); *, significantly different from control, $*p \leq 0.05$. This experiment is representative of three independently performed experiments.