

The imbalanced expression of matrix metalloproteinases in nephrogenic systemic fibrosis

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Background: Nephrogenic systemic fibrosis (NSF) occurs in patients with renal dysfunction and gadolinium exposure. Although little is known about the pathogenesis of this disease, increased expression of transforming growth factor- β has been recently demonstrated. Other fibrosing conditions have been shown to express an imbalance in matrix metalloproteinase (MMP) expression and their corresponding inhibitors. Myofibroblast differentiation, in which cells often express α -smooth muscle actin and achieve the ability to contract, is also a hallmark of fibrosis.

Objective: We theorized that NSF may overexpress tissue inhibitor of metalloproteinase-1 (TIMP-1), while simultaneously showing decreased expression of MMP-1. As a secondary aim, we sought to evaluate the presence of smooth muscle actin in our samples.

Methods: We applied immunohistochemistry to 16 skin biopsies from 10 patients with NSF using antibodies to TIMP-1, MMP-1, MMP-2, MMP-9, and α -smooth muscle actin. Samples from normal skin, scar, keloid and scleroderma were stained for comparison.

Results: TIMP-1 was strongly expressed in all NSF specimens compared to normal skin. MMP-1 expression was nearly absent in all tested samples. In all 16 NSF cases, the dermal spindle cells did not stain for α -smooth muscle actin. MMP-2 and MMP-9 expression was variable but was increased compared to normal skin.

Limitations: The expression is semiquantitative and based on immunohistochemistry and unconfirmed by other techniques.

Conclusions: In NSF, TIMP-1 is strongly expressed and MMP-1 is nearly absent, characteristic of the MMP imbalances seen in other fibrosing processes. Using smooth muscle actin immunohistochemistry, there was no evidence of myofibroblast differentiation. (J Am Acad Dermatol 2010;63:483-9.)

Key words: matrix metalloproteinase; myofibroblast; nephrogenic fibrosing dermatopathy; nephrogenic systemic fibrosis; smooth muscle actin; tissue inhibitor of matrix metalloproteinase; transforming growth factor.

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Conflicts of interest: None.

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INTRODUCTION

Nephrogenic systemic fibrosis (NSF), known formerly as nephrogenic fibrosing dermatopathy, is a fibrosing condition strongly associated with renal dysfunction and exposure to gadolinium-containing contrast agents used in imaging studies.¹⁻⁴ The skin is characterized by extensive thickening and hardening associated with brawny hyperpigmentation (Fig 1). Systemic involvement has been documented, including fibrosis of striated muscle, myocardium, lungs, dura mater, renal tubules and rete testes.^{5,6} Since 1997, more than 315 patients with renal disease and NSF have been registered in the NSF registry at Yale University.⁷

Histologic features reveal a dermis containing thickened collagen bundles, variable mucin, and an

increased number of spindled and plump fibroblast-like cells. In some cases there are small multinucleated histiocytes. In deep biopsy samples, the process may extend beyond the dermis and permeate the septa of subcutaneous fat lobules. There are minimal to absent inflammatory cells⁸ (see Fig 1). The spindle-shaped cells co-express CD34 (see Fig 1) and procollagen-1, leading many to believe their origins to be from bone marrow–derived circulating fibrocytes.⁹ Despite isolated case reports for many potential therapies, including ultraviolet A1 radiation,¹⁰ extracorporeal photopheresis,¹¹ and more recently imatinib,¹² there is no universally effective or standard treatment for the disease.

The pathogenesis of NSF is unclear, but leading theories surround the stimulation of bone marrow–derived CD34-positive circulating fibrocytes by an unknown mechanism and their accumulation in affected tissue.⁹ Gadolinium has been detected in affected fibrotic tissue of NSF,^{3,4,13} but it is not clear if or how this may stimulate fibrosis. Regardless, fibrosis progresses and pro-fibrotic cytokine expression of transforming growth factor-beta (TGF- β), as well as its second messenger, SMAD, have been previously shown by us¹⁴ and others.¹⁵

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endoproteinases that are essential for various normal biological processes such as embryonic development, morphogenesis, reproduction, tissue resorption, and remodeling.¹⁶ Tissue inhibitor of metalloproteinase 1 (TIMP) is an important inhibitor of MMPs. TIMP and MMPs constitute a tightly regulated system in normal wound healing, and their imbalance has been implicated in pathologic fibrosing diseases of the skin, including hypertrophic scars and keloids as well as scleroderma.¹⁷⁻¹⁹ Elevated TIMP relative to MMP expression has been described in serum and skin fibroblasts from patients with systemic sclerosis.¹⁴⁻¹⁶ Increased TIMP and decreased MMP-1 expression in human fibroblasts has also been shown to occur in the presence of TGF- β .²⁰⁻²³ Hence, since we and others^{11,12} have previously shown TGF- β expression in NSF, we theorized that NSF would show this same pattern of increased TIMP and decreased MMP-1.

In addition, we reasoned that some of the spindle cells would show evidence of myofibroblast differentiation since the presence of this cell is a key feature of normal wound healing as well as pathologic scar formation and chronic fibrosing conditions including scleroderma^{24,25} and is also known to be stimulated by TGF- β .²⁶

To investigate these aims, we applied immunohistochemistry to paraffin-embedded tissues using antibodies to TIMP-1, MMP-1, MMP-2, MMP-9, and smooth muscle actin (SMA). Sixteen specimens from 11 patients with NSF from our dermatopathology files were included in our analysis. Samples of normal skin, scar, keloid, and scleroderma were stained for comparison.

MATERIAL AND METHOD

Sixteen formalin-fixed, paraffin-embedded specimens from 10 patients with NSF from the University of

Texas Medical Branch were included in our analysis. Eight of these 10 patients (patients A through H) were included in our previous study¹¹ (Table I).

Sections 4- to 6- μ m thick were applied to slides, deparaffinized in xylene, hydrated through graded alcohols, and washed. Antigen unmasking was performed via heat treatment for 20 minutes with 10 mmol/L sodium citrate buffer at 95°C. The slides were allowed to cool for 20 minutes, then rinsed in distilled water and placed into a container of TRIS Buffered Saline with Tween 20 (Signet Pathology Systems, Inc, Dedham, MA; Catalog No. 80). Automated immunohistochemistry was then performed with the Dako Autostainer (Carpinteria, CA). Before labeling with primary antibody to prevent nonspecific reactions, sections were incubated with blocking reagent from Avidin Biotin Blocking Kit (Vector Laboratories, Burlingame, CA; Catalog No. SP2001) for 7 minutes. The following primary antibodies were used: TIMP-1 at a 1:50 dilution (sc-21734, Santa Cruz Biotechnology), MMP-1 at a 1:50 dilution (sc-21731, Santa Cruz Biotechnology), MMP-2 at a 1:100 dilution (sc-58386, Santa Cruz Biotechnology), MMP-9 at a 1:100 dilution (sc-21733, Santa Cruz Biotechnology), and α -smooth muscle actin (Dako M0851) at a dilution of 1:200. Primary antibodies incubated for 30 minutes, secondary

CAPSULE SUMMARY

- Nephrogenic systemic fibrosis (NSF) is a fibrosing condition that occurs in patients with renal dysfunction and gadolinium exposure in which little is known about the mechanism of fibrosis.
- By immunohistochemistry, we demonstrate strong expression of TIMP-1 accompanied by near absent expression of MMP-1, characteristic of matrix metalloproteinase imbalances seen in other fibrosing processes.
- NSF samples were negative for α -smooth muscle actin, suggesting a lack of myofibroblastic differentiation in this disorder.

Abbreviations used:

MMP:	matrix metalloproteinase
NSF:	nephrogenic systemic fibrosis
SMA:	smooth muscle actin
TGF- β :	transforming growth factor- β
TIMP:	tissue inhibitor of metalloproteinase

antibodies incubated for 15 minutes. Slides were then counterstained with hematoxylin, washed with deionized water, dehydrated through graded alcohols and xylene, and finally coverslipped.

For TIMP-1 and the MMPs, dilutions were appropriately titrated using controls of thyroid and liver per manufacturer's recommendations. Negative controls were placenta, lung, and kidney.

For comparison to other fibrosing conditions, normal skin, a 13-day-old skin scar, and localized scleroderma were stained in the same manner with the MMPs and TIMP-1. Normal skin, keloid, and localized scleroderma were also stained with smooth muscle actin.

Immunohistochemical stains were evaluated for the presence of positively staining fibroblast-like spindle cells between collagen bundles in the dermis. The following semiquantitative scale, based on percentage of positively staining spindle cells, was used: - (no staining), + (<25% staining), ++ (25%-50% staining), +++ (50%-75% staining), and ++++ (75%-100% staining).

RESULTS

Table I shows clinical information and immunohistochemical staining results from the 16 skin biopsy specimens from 10 patients with NSF, as well as the samples used for comparison.

TIMP-1 was found to be strongly expressed in the cytoplasm of spindle cells in all NSF specimens (Fig 2), with 11 of 16 biopsies showing 4+ staining (average 3.6+). Both the scar and scleroderma samples stained strongly as well, with 4+ staining. In normal skin, TIMP-1 was 1+.

MMP-1 was completely absent in 13 of 16 NSF samples; when present, it was only weakly expressed with 1+ staining. There was no staining for MMP-1 in scar, scleroderma, or normal skin (see Fig 2).

NSF and the other fibrotic conditions (scar, localized scleroderma) showed more variable expression of MMP-2 and MMP-9 (see Fig 2). The average MMP-2 and MMP-9 expression in NSF samples was 2.3+ and 3.4+, respectively. Normal skin was negative for both MMP-2 and MMP-9.

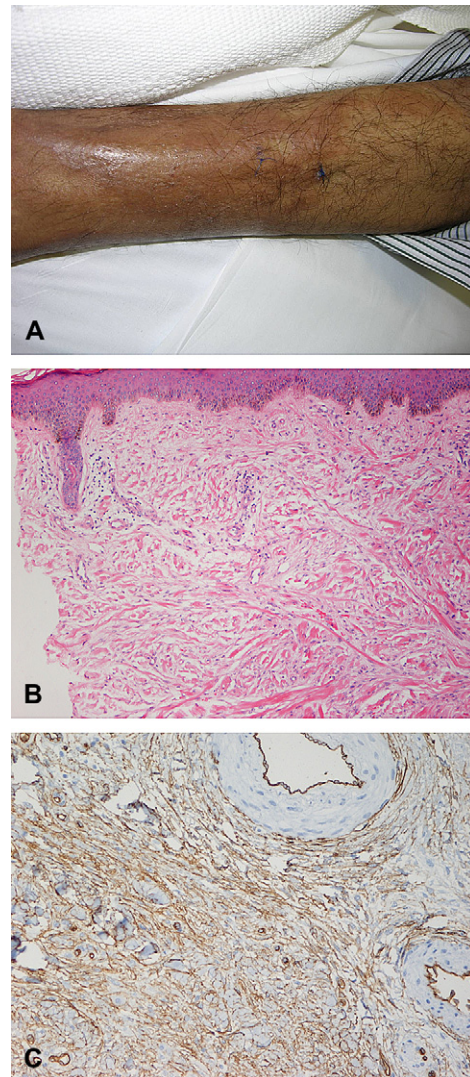


Fig 1. **A**, Indurated, hyperpigmented plaque of NSF on lower extremity. **B**, Increased fibroblast-like spindle cells between thick collagen bundles. (Hematoxylin-eosin stain; original magnification $\times 40$.) **C**, Spindle cells stain positive with CD34.

All but one of our patients underwent biopsy less than a year after exposure to gadolinium. One underwent biopsy 3.8 years after exposure (Patient I) and showed a persistent unbalanced expression of TIMP and MMPs similar to those patients who underwent biopsy earlier in the course of the disease.

In all 16 cases, no α -smooth muscle actin staining was seen in the spindle cells (Fig 3). Myoepithelial cells in vessels served as internal controls. In contrast, localized scleroderma and keloid revealed spindle cells staining positive for SMA (see Fig 3).

DISCUSSION

In normal wound healing, injured tissue is filled with blood cells and fibrin, followed by the release of

Table I. Expression of matrix metalloproteinases and tissue inhibitor of metalloproteinase in nephrogenic systemic fibrosis samples

Patient	Specimen No.	Age (y)/Race/Sex	Time from Gd exposure to biopsy	Time from symptom onset to biopsy	TGF- β *	TIMP	MMP-1	MMP-2	MMP-9	SMA
A	1	33/AA/F	4 mo	2 mo	—	+++	+	+++	+++	—
	2				+++	++	—	+	+++	—
B	3	62/AA/F	7 mo	7 mo	—	+++	—	++	+++	—
C	4	42/LA/M	1-2 mo	1 mo	++	++++	+	+	++++	—
D	5	14/LA/F	4-5 mo	2 mo	+	++++	—	++	+++	—
	6				+	++++	—	++++	++++	—
E	7	38/LA/F	4 mo	1 mo	—	++++	—	+++	++++	—
	8				—	++++	—	+++	+++	—
F	9	87/W/M	2 mo	2 wk	+++	++++	—	+	+++	—
	10				+++	++++	—	++	++++	—
G	11	56/Asian/M	3 mo	3 mo	—	++++	—	+++	++++	—
	12				++	+++	—	+	++++	—
H	13	37/AA/F	Unknown	Unknown	+	++++	—	++++	+++	—
I	14	56/AA/F	46 mo	Unknown	NP	+++	+	+++	+++	—
	15			Unknown	NP	++++	—	++	+++	—
J	16	45/LA/M	8 mo	6 mo	NP	++++	—	+++	+++	—
Controls										
Normal skin					NP	+	—	—	—	NP
13-day-old scar					NP	++++	—	++++	+	NP
Localized scleroderma					NP	++++	—	+	—	+
Keloid					NP	NP	NP	NP	NP	+

AA, African American; F, female; Gd, gadolinium; LA, Latino/Latina American; M, male; NP, not performed; W, white.

*Data previously published.¹⁴

various growth factors. Next, proliferation of fibroblasts, smooth muscle cells, and endothelial cells leads to formation of granulation tissue. Tissue regeneration is then initiated and progresses until wound repair is complete.^{27,28} To prevent fibrosis, matrix homeostasis must be tightly balanced to avoid excessive production or decreased degradation of collagen and the extracellular matrix.

MMPs, in addition to their many other functions, play an important role in wound healing. After injury, MMP-1, -2 and -9 expression is rapidly increased and gradually declines as the wound enters the remodeling phase. MMP-1, also called interstitial collagenase, has the distinctive capacity to cleave the triple helix of type I collagen, allowing the chains to unwind and become susceptible to further degradation.²⁹ MMP-2 and MMP-9 are gelatinases which degrade type IV collagen, a major constituent of basement membranes, denatured interstitial collagens (gelatins), laminin, elastin, and fibronectin.³⁰ The system is tightly regulated by TIMPs. Continued expression of TIMP-1 would be expected to inhibit the ability of MMPs, particularly MMP-1, to appropriately break down the extracellular matrix resulting in fibrosis. In fact, this has been shown in animal models as well as in patients with hypertrophic scars and scleroderma.¹⁴⁻¹⁶

Myofibroblasts also appear to play a key role in the process of wound healing. Not only do they synthesize extracellular matrix components such as collagen types I-IV, proteoglycans, and matrix-modifying proteins, they also promote contraction of the granulation tissue by virtue of their expression of the contractile protein α -smooth muscle actin. During wound healing, the myofibroblasts are eliminated by apoptosis. This process must be tightly regulated, as overactivation of myofibroblasts is responsible for fibrosis and scarring and has been shown in many fibrotic diseases, including hypertrophic scars developing after burn injury, fibrotic phase of scleroderma^{21,31} and in the palmar fibromatosis of Dupuytren's disease as well as fibrosis affecting vital organs such as the liver,³² heart,³³ lung,³⁴ and kidney.³⁵

Compelling evidence of the importance of myofibroblasts in scarring may have best been demonstrated in fibroblasts from scleroderma patients. Both unaffected and scarred skin samples showed increased expression of profibrotic cytokines, but only the scarred areas had increased ability to adhere and contract extracellular matrix. This suggests that increased extracellular matrix per se may not be enough to result in scar. Rather, mechanical tension may also be required.²⁴

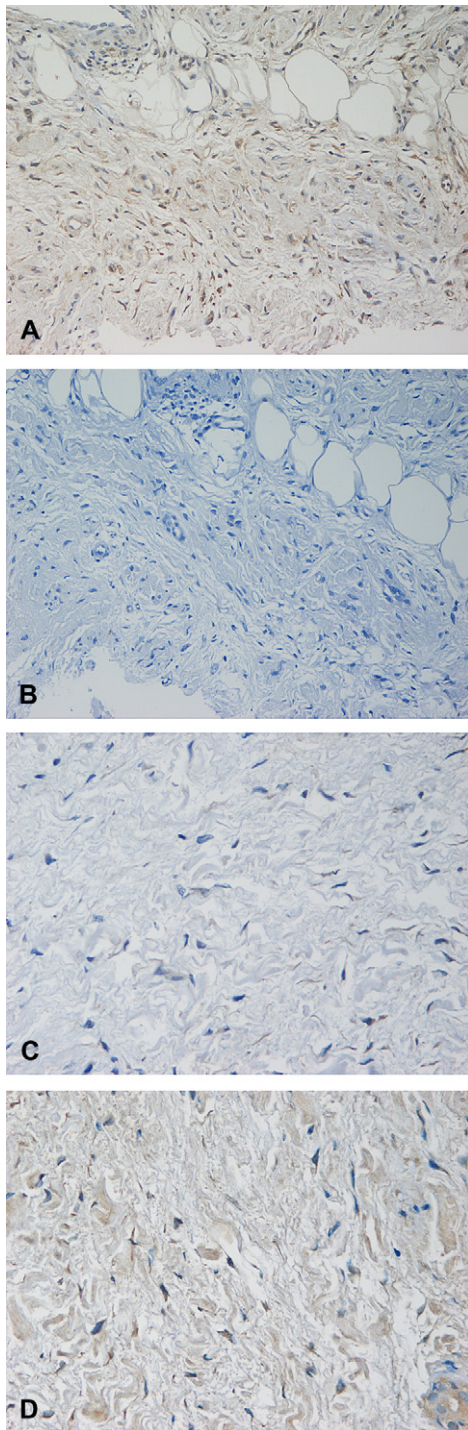


Fig 2. **A**, Cytoplasm of spindle cells in NSF stain strongly with TIMP-1. **B**, Spindle cells are negative for MMP-1. More variable staining was seen with **(C)** MMP-2 and **(D)** MMP-9.

The major finding in our study is that expression of TIMP-1 is strongly expressed in the spindle cells of NSF. Additionally, we found essentially absent MMP-1 staining in all NSF samples. This ratio of increased

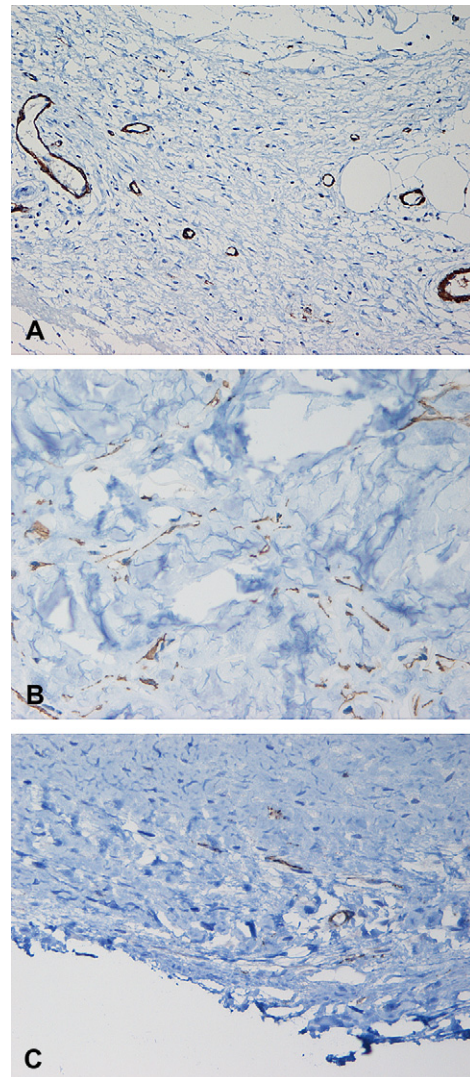


Fig 3. **A**, Spindle cells of NSF do not stain for SMA. Focal areas of SMA-positive spindle cells are seen in **(B)** scleroderma and **(C)** keloid.

TIMP and decreased MMP-1 might be expected to result in fibrosis and, in fact, has been shown in scleroderma and keloids.³⁶ This imbalance was present in all of our NSF samples, even in a sample that was taken nearly 4 years after the onset of symptoms. It is not known why this imbalance would persist, but perhaps the continued presence of TGF- β , which is known to stimulate TIMP-1²⁰ and which we have previously shown in NSF lesions,¹¹ plays a role.

A second and unexpected finding is that we found no evidence of α -smooth muscle actin expression in any of the spindle cells of all of our NSF samples. We did, however, find α -smooth muscle actin staining in both the keloid and scleroderma control samples as evidence of myofibroblastic differentiation.

One major difference between NSF and other fibrosing conditions such as scleroderma is the

prominence of CD34+ expression. Some have speculated that this signifies a bone-marrow derivation of the spindle cell as opposed to local recruitment of tissue and proliferation fibroblasts. These cells have been called circulating fibrocytes. Fibrocytes isolated from the peripheral blood of healthy people have been shown to differentiate into myofibroblasts and express α -SMA when TGF- β is present through the activation of second messengers SMAD 2/3 and SAPK/JNK MAPK pathways.²⁵ These spindle cells (fibrocytes) from NSF have been shown to express SMA when grown in cell culture.³⁷ It is therefore unclear why our samples of paraffin-embedded tissue do not show expression of SMA despite the documented association of TGF- β and its second messenger SMAD with the disease. Because myofibroblasts undergo apoptosis during scar resolution,³⁸ it is possible that the cells were present initially in the NSF lesions but eventually underwent cell death during the latter stages of fibrosis. In fact, at least one study has shown that myofibroblast apoptosis was dependent on MMP-2 and MMP-9 expression, which was also present in our NSF samples.³⁹ Nevertheless, this seems unlikely given that the fibrosing process in many of these patients was still progressing clinically and the length of time from symptom onset to biopsy varied from 2 weeks to 4 years. It may also be that our immunohistochemical techniques were not sensitive enough to detect myofibroblast differentiation and that molecular-based methods, ultrastructural studies or that other markers, such as calponin and caldesmon, would be able to identify the presence of contractile elements in the spindle cells.³⁸ A third possibility is that other cytokines or mechanical factors inhibit the differentiation of these cells into myofibroblasts. Tumor necrosis factor alpha, interferon gamma,⁴⁰ basic fibroblast growth factor,⁴¹ and interleukin 1⁴² have all been shown to inhibit SMA expression in the presence of TGF- β .

Regarding potential future therapies, knowledge of the pathogenesis of fibrosis may be helpful in determining targets. Broadly targeting TGF- β signaling for the treatment of disease is anticipated to be problematic, largely on the basis of findings from animal studies. For example, TGF- β -deficient mice display a severe wasting syndrome accompanied by a generalized inflammatory response and tissue necrosis, resulting in organ failure and death.^{43,44} Animals genetically deficient in TGF- β receptor type I (ALK5) die in utero and display severe vascular defects.⁴⁵ Mice deficient in SMAD 3, a second messenger of TGF- β , become moribund with chronic inflammation and colorectal adenocarcinomas between 4 and 6 months of age⁴⁶ and can also develop

degenerative joint diseases.⁴⁷ Perhaps better targets of therapy will be the induction of MMPs or destruction of their inhibitors, like TIMP, which may operate in tandem or downstream of TGF- β in the fibrotic pathway. Interestingly, some anti-fibrotic properties have been shown using an antisense oligonucleotide against TIMP in rats with liver fibrosis.⁴⁸

In conclusion, by using immunohistochemistry on paraffin-embedded tissue from patients with nephrogenic systemic fibrosis, we found that TIMP-1 is strongly expressed while MMP-1 is largely absent, similar to findings previously reported in other profibrotic conditions. No α -smooth muscle actin-positive spindle cells were seen in our NSF samples, which suggests that myofibroblasts are not present in this fibrosing disorder.

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