Published in final edited form as:

Invest Radiol. 2010 January ; 45(1): 42. doi:10.1097/RLI.0b013e3181bf95eb.

Collagenolytic Activity is Suppressed in Organ-Cultured Human Skin Exposed to a Gadolinium-Based MRI Contrast Agent

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Abstract

Objective—Human skin produces increased amounts of matrix metalloproteinase-1 (MMP-1) when exposed in organ culture to Omniscan, one of the gadolinium-based MRI contrast agents (GBCA). MMP-1, by virtue of its ability to degrade structural collagen, contributes to collagen turnover in the skin. The objective of the present study was to determine if collagenolytic activity was concomitantly upregulated with increased enzyme.

Materials and Methods—Skin biopsies from normal volunteers were exposed in organ culture to Omniscan. Organ culture fluids obtained from control and treated skin were examined for ability to degrade type I collagen. The same culture fluids were examined for levels of MMP-1, tissue inhibitor of metalloproteinases-1 (TIMP-1), and complexes of MMP-1 and TIMP-1.

Results—Although MMP-1 was increased in culture fluid from Omniscan-treated skin, there was no increase in collagenolytic activity. In fact, collagenolytic activity declined. Increased production of TIMP-1 was also observed in Omniscan-treated skin, and the absolute amount of TIMP-1 was greater than the amount of MMP-1. Virtually all of the MMP-1 was present in MMP-1–TIMP-1 complexes, but the majority of TIMP-1 was not associated with MMP-1. When human dermal fibroblasts were exposed to TIMP-1 (up to 250 ng/ml), no increase in proliferation was observed, but an increase in collagen deposition into the cell layer was seen.

Conclusion—GBCA exposure has recently been linked to a fibrotic skin condition in patients with impaired kidney function. The mechanism is unknown. The increase in TIMP-1 production and concomitant reduction in collagenolytic activity demonstrated here could result in decreased collagen turnover and increased deposition of collagen in lesional skin.

Keywords

collagenolytic activity; gadolinium-based contrast agent (GBCA); matrix metalloproteinase I (MMP-1); nephrogenic systemic fibrosis (NSF); tissue inhibitor of metalloproteinases-1 (TIMP-1)

INTRODUCTION

Nephrogenic systemic fibrosis (NSF) is a clinical syndrome linked in individuals with renal failure to exposure to gadolinium-based contrast agents (GBCAs) during magnetic resonance imaging (MRI) procedures (1–10). The disease has been likened to scleroderma, but most pathologists describe the lesions as fibroplastic (1,2,8,11,12), based on the presence of numerous, “plump,” fibroblast-like cells and a mucin-rich matrix.

The mechanistic events that lead from GBCA exposure to NSF are not understood. Delineating the critical patho-physiological events in NSF has been slow, at least in part because there are no models that precisely mimic the disease. Recently, Sieber et al (13,14) showed that some of the features of NSF could be seen in healthy rats following repeated injection with various chelated gadolinium moieties. Inflammation is common in end-stage renal disease (15) and *in vitro* studies have shown that human monocytes respond to direct stimulation with GBCAs by elaborating pro-inflammatory cytokines (16). Other *in vitro* studies have demonstrated increased fibroblast proliferation in response to chelated gadolinium compounds (17). In a recent study, we showed that exposure of human skin in organ culture or human dermal fibroblasts in monolayer culture to any of several clinically used GBCAs resulted in increased elaboration of both matrix metalloproteinase-1 (MMP-1) and tissue inhibitor of metalloproteinases-1 (TIMP-1) without an increase in type I procollagen production (18). Based on these observations, it was suggested that the response to GBCA stimulation might involve alterations in an enzyme-inhibitor system that regulates collagen turnover in the skin rather than an effect on collagen production, *per se*.

The present study continues our effort to understand how GBCA exposure initiates fibro-proliferative / fibrotic changes in the skin of susceptible individuals. Here we show that although MMP-1 levels are significantly elevated in GBCA-treated skin, there is essentially no collagenolytic activity. Our data indicate that the lack of collagenolytic activity is due, at least in part, to increased TIMP-1 production and inactivation of MMP-1 by the metalloproteinase inhibitor. In addition to the increase in TIMP-1, the chelators associated with gadolinium in the GBCAs also directly inhibit collagenolytic activity. To the extent that suppression of collagenolytic activity contributes to NSF (not known at present), these findings may be relevant to understanding disease pathophysiology.

MATERIALS AND METHODS

Reagents

Omniscan (GE Healthcare), Magnevist (Berlex Imaging), and Multihance (Bracco diagnostics) were obtained from the In-Patient Pharmacy at the University of Michigan Hospitals as ready-for-use solutions of chelated gadolinium (0.5M with respect to the active moieties). Gadolinium chloride was obtained from Sigma Chemical Company (St. Louis, MO).

Human MMP-1 (collagenase-1) was obtained from Calbiochem. The enzyme was purified from human rheumatoid synovial fibroblasts as the naturally occurring proenzyme form. The MMP-1 preparation was reactive with a rabbit polyclonal anti-MMP-1 antibody (AB806; Chemicon) and appeared as a doublet at 52 and 57 kD in Western blots. Human recombinant TIMP-1 was obtained from Calbiochem.

Human skin organ culture

In a previous study (18), we obtained replicate 2-mm punch biopsies of sun-protected hip skin from normal subjects. Tissue acquisition was approved by the Institutional Review Board (IRB) at the University of Michigan, and all subjects provided written informed consent prior to inclusion in the study. The skin biopsies were incubated in wells of a 24-well dish (one tissue

piece per 500 μ L of culture medium). Culture medium consisted of Keratinocyte Basal Medium (KBM) (Lonza), a low- Ca^{2+} (0.10 mM) modification of MCDB-153 medium. For our purposes, the culture medium was supplemented with calcium chloride to achieve a final Ca^{2+} concentration of 1.55 mM. One or two tissue pieces were left as control while the others were treated with Omniscan over a range of concentrations. In some cases, gadolinium chloride was used in place of Omniscan. Organ culture-conditioned medium was collected at Day 3. The serum-free, growth factor-free culture medium was assessed for collagenolytic activity as well as for MMP-1, TIMP-1, and complexes of MMP-1–TIMP-1 as described below.

Collagenolytic activity

Collagen degradation by enzymes present in skin organ culture fluid was assessed as aforementioned (19). Rat tail collagen (3.7 – 4.7 mg/mL in 1N HCl) (BD Biosciences) was diluted to 2 mg/mL in culture medium consisting of serum-free, Ca^{2+} -supplemented KBM. The collagen solution was made isotonic by addition of an appropriate amount of 10X concentrated Hanks' Balanced Salt Solution and brought to pH 7.2. Degradation of the polymerized collagen was achieved by exposing the collagen to human skin organ culture fluid or, in some experiments, to human synovial fibroblast-derived MMP-1. When fibroblast-derived enzyme was utilized, activation of the proenzyme was accomplished by exposure of the latent enzyme to 1 μ g of crystalline trypsin for 5 minutes at 37°C followed by 10 μ g of soybean trypsin inhibitor (SBTI) to neutralize residual trypsin. Intact collagen exposed to buffer alone served as a negative control. Organ culture fluid from a basal cell tumor was used as a positive control. After enzyme treatment (18 hours at 37°C), collagen fragmentation was assessed by measuring the peptides (specifically, the $\frac{1}{4}$ - and $\frac{3}{4}$ -sized fragments) released into the supernatant fluid. SDS-PAGE resolution and staining with Coomassie brilliant blue was used for this. The resolved bands were digitized and quantified.

MMP-1

Western blotting with a rabbit polyclonal anti-MMP-1 antibody was used to assess MMP-1 levels (18). Briefly, samples were separated in SDS-PAGE under denaturing and reducing conditions and transferred to nitrocellulose membranes. After blocking with a 5% nonfat milk solution in Tris-buffered saline with 0.1% Tween (TTBS) at 4°C overnight, membranes were incubated for one hour at room temperature with the antibody, diluted 1:1000 in 0.5% nonfat milk/0.1% TTBS. Thereafter, the membranes were washed with TTBS and bound antibody detected using the Phototope-HRP Western blot detection kit (Cell Signaling Technologies, Inc.). Images were scanned, digitized, and quantified (both latent and active forms). Prior to loading the gels, protein levels in each sample were determined using the BCA protein determination kit (Pierce Biotechnology) and equal amounts of protein were loaded onto each lane. Following electrophoresis and protein transfer to the nitrocellulose filters, Ponceau S reversible staining solution (Pierce Biotechnology) was used to visualize the transferred proteins and to confirm that comparable amounts of total protein were transferred.

TIMP-1

Culture fluids were assayed for TIMP-1 by ELISA using a commercially available assay kit (R&D Systems) (20,21).

MMP-1–TIMP-1 complex

Culture fluids were assayed for MMP-1–TIMP-1 complexes by ELISA using a commercially available assay kit (R&D Systems). The assay is similar to the TIMP-1 ELISA but measures a complex of the two proteins.

Human dermal fibroblasts

Human dermal fibroblasts were obtained from skin biopsies as previously described (22). The isolated cells were grown using Dulbecco's Modified Minimal Essential Medium supplemented with nonessential amino acids and 10% fetal bovine serum (DMEM-FBS) as culture medium. Maintenance was at 37°C in an atmosphere of 95% air and 5% CO₂. Cells were used at passage 2–3.

Assessment of fibroblast proliferation and type I collagen deposition in response to TIMP-1

Fibroblasts were harvested from 75-cm² culture flasks by brief (5-minute) exposure to a solution of 0.05% trypsin with 0.2 mg/mL ethylenediaminetetraacetic acid (EDTA). The cells were counted and added to wells of a 24-well dish at 2×10^4 cells per well in DMEM-FBS. The cells were allowed to attach and spread. They were then washed two additional times with 1 mL of serum-free, Ca²⁺-supplemented Keratinocyte Growth Medium (KGM) (Lonza). KGM contains the same basal medium as KBM but is supplemented with EGF, insulin, and pituitary extract. Duplicate wells were counted to provide zero-time values. The rest of the wells were used in the experiment. TIMP-1 was added at the desired concentration in a 0.5 mL volume, and the cells were incubated for 3 days at 37°C in an atmosphere of 95% air and 5% CO₂. At the end of the incubation period, culture fluids were collected and cells were harvested with trypsin-EDTA and counted.

In other experiments, cells were added to wells of a 6-well dish at 2×10^5 cells per well. Cells were treated with TIMP-1 as aforementioned. However, instead of harvesting and counting cells at the end of the 3-day incubation period, the cell layer was washed one time in PBS and then treated with M-PER, a proprietary, detergent-containing mammalian protein extraction agent (Pierce Biotech) according to the manufacturer's instructions. As described above, Type I collagen was assessed in the detergent lysates by Western blotting with MMP-1 except that non-denaturing and non-reducing conditions were used. The antibody was a rabbit polyclonal antibody (Abcam, ab292) that detects bands between 80 – 120 kD.

Statistical analysis

Data were analyzed using the Student t-test or using one-way analysis of variance (ANOVA) followed by the Bonferroni post-test for selected pairs (GraphPad Prism 4.0 for Windows, GraphPad Software). Data were considered significant at $p < 0.05$. Asterisks have been added to the appropriate bars in each figure to denote values that are significantly different from the respective control values.

RESULTS

Collagenolytic activity in Omniscan-treated human skin

In a previous study, 2-mm punch biopsies of sun-protected hip skin from several normal human donors were treated in organ culture with Omniscan over a wide range of concentrations (18). The serum-free, growth factor-free culture fluids were obtained from control and treated tissue at Day 3. In the first series of experiments presented here, we examined these organ culture fluids for collagenolytic activity, i.e., for capacity to cleave intact type I collagen into 1/4- and 3/4-sized fragments. Collagen cleavage was obtained with organ culture fluid from control skin. While clearly detectable (Figure 1 inset), the level of collagen-cleaving activity was low compared to what we have observed previously using organ-cultured skin from basal cell carcinomas (23,24) or with normal skin following acute exposure to ultraviolet light (25–27). The inset demonstrates the fragmentation pattern observed with culture fluid from the basal cell tumor. Multiple fragments are observed because the 1/4- and 3/4-sized fragments are susceptible to further digestion. Most importantly, it can be seen from Figure 1 that there was

less collagen-cleaving activity in culture fluids from tissue treated with Omniscan (50 μ M) during the three day incubation period. Culture fluids from tissue treated with higher Omniscan concentrations (250 μ M to 2.5 mM) were completely devoid of collagenolytic activity (not shown).

As part of the initial set of experiments, organ culture fluid from control and Omniscan-treated skin was examined for excess inhibitory activity. Human synovial fibroblast-derived MMP-1 was activated with trypsin as described in the Materials and Methods section. The activated enzyme was then exposed to organ culture fluid from untreated and Omniscan-treated skin. Following this, collagen digestion by enzyme alone and by the enzyme pre-incubated with organ culture fluid was assessed. Collagen-digestion was reduced following pre-incubation of the activated MMP-1 with culture fluid from either control tissue or Omniscan-treated tissue. Organ culture fluid from tissue treated with Omniscan had greater residual inhibitory capacity. Specifically, under conditions in which control skin organ culture fluid inhibited MMP-1-driven collagenolytic activity by 29 ± 4 %, there was 74 ± 12 % inhibition with culture fluid from Omniscan-treated skin (mean and standard deviation; $n=3$ experiments; $p<0.05$).

MMP-1, TIMP-1, and MMP-1–TIMP-1 complexes in organ culture fluid

MMP-1 and TIMP-1 concentrations were assessed in organ culture fluids (based on Western blotting and ELISA, respectively). Consistent with findings in our past report (18), MMP-1 was elevated in culture fluid from tissue exposed to Omniscan as compared to control (upper panel). An increase in active as well as latent forms of the enzyme was observed (inset). As part of our analysis, MMP-1 purified from synovial fibroblasts (commercially available) was used to generate a standard curve. When MMP-1 in the organ culture fluids from three different experiments was compared to the standard curve, the amounts present in control organ culture fluid was 1–2 ng/mL (18–36 pM), while the calculated amounts present in the culture fluid from Omniscan-treated skin was 5–6 ng/mL (90–108 pM).

The middle panel of Figure 2 presents results from analyses for TIMP-1. Culture fluids from Omniscan-treated tissue contained elevated amounts of TIMP-1 relative to control. In absolute amounts, the levels of TIMP-1 were significantly higher than the levels of MMP-1 (up to 1500 pM as compared to approximately 100 pM for MMP-1). While a 50 μ M concentration of Omniscan was used in this experiment, our previous study (18) demonstrated that increased TIMP-1 could be seen in organ culture fluid from tissue exposed to the GBCA at concentrations as high as 0.5 – 2.5 mM.

The lower panel of Figure 2 shows results from studies in which MMP-1–TIMP-1 complexes were assessed. The amount of enzyme-inhibitor complex was higher in culture fluid from Omniscan-treated skin than in culture fluid from control skin. Not surprisingly, in light of the above findings, the total amount of complex appeared to reflect the amount of available MMP-1 more than TIMP-1.

Direct inhibition of collagenolytic activity with Omniscan

GBCAs consist of gadolinium coupled to a metal ion chelator. Since metalloproteinases are dependent on Zn^{2+} and Ca^{2+} for activity, it is possible that the GBCAs themselves directly interfere with MMP-driven collagenolytic activity. To test for this, Omniscan was examined for direct inhibition of MMP-1-mediated collagen digestion. Human synovial fibroblast-derived MMP-1 was activated with trypsin as above and exposed to Omniscan over a wide range of concentrations. Following this, collagen digestion by the enzyme alone as well as by the enzyme pre-incubated with the GBCA was assessed. Collagen digestion was reduced following pre-incubation of the activated MMP-1 with Omniscan. Effective inhibition was observed at 500 μ M and above. There was essentially no decrease in collagenolytic activity at

50 μM concentrations or below (Figure 3). In additional experiments (not shown), two other GBCAs (e.g., Magnevist and Multihance) were also found to suppress MMP-1 collagenolytic activity at high concentrations (500 μM and above) but did not suppress activity at lower concentrations.

Inhibition of collagenolytic activity with organ culture fluid from gadolinium chloride-treated skin

As an additional control for chelator effects, organ culture fluids were obtained from tissues that had been exposed to gadolinium chloride (10–50 μM) for three days in place of the GBCA. Previous studies have demonstrated that fibroblast proliferation is stimulated by these concentrations of gadolinium chloride (18), and in the present study, we confirmed increased TIMP-1 in organ culture fluids from gadolinium chloride-treated skin as compared to culture fluid from control skin. There was 15 ± 8 ng/mL of TIMP-1 in the control culture fluid versus 43 ± 6 and 65 ± 29 ng/mL in culture fluid from skin exposed to 10 and 50 μM gadolinium chloride. Activated MMP-1 was exposed to organ culture fluid from control or gadolinium chloride-treated skin and residual collagenolytic activity assessed as above. As can be seen in Figure 4, MMP-1-driven collagenolytic activity was reduced in the presence of control skin organ culture fluid and to a greater extent in the presence of organ culture fluid from gadolinium chloride-treated skin.

Effects of exogenous TIMP-1 on human dermal fibroblast proliferation and type I collagen deposition in monolayer culture

In a final set of experiments, exogenous human recombinant TIMP-1 was examined for ability to stimulate fibroblast proliferation. The results of this study are shown in the upper panel of Figure 5, where it can be seen that fibroblast proliferation was unaffected by TIMP-1 concentrations between 10 and 250 ng/mL. Although there was no increase in proliferation under the conditions utilized here, there was an increase in deposition of type I collagen into the cell layer / extracellular matrix in Omniscan-treated fibroblasts as compared to control (Figure 5, lower panel).

DISCUSSION

The present study continues our effort to understand how the chelated-gadolinium compounds influence collagen metabolism in the skin. Here we show that although the level of MMP-1 is significantly elevated in Omniscan-treated skin (Figure 2 and ref. 18), collagenolytic activity is not increased commensurately. Rather, the level of collagen-degrading activity is actually decreased as compared to the low level present in untreated skin.

The decrease in collagenolytic activity appears to be due, at least in part, to increased TIMP-1 production and inactivation of MMP-1 by the inhibitor. In 3-day organ culture fluid from Omniscan-treated tissue, the level of TIMP-1 was increased approximately 2-fold relative to the level seen in culture fluid from control skin. While MMP-1 was also increased, the absolute amount of TIMP-1 was much greater than that of MMP-1, i.e., approximately 40 ng/mL (1500 pM) for TIMP-1 versus approximately 5–6 ng/mL (100 pM) for MMP-1. When MMP-1–TIMP-1 complex formation was assessed in the culture fluid from Omniscan-treated skin, the amount of protein in the complexes (3.2 ng/mL) was found to reflect the total amount of MMP-1 in the culture fluid rather than the amount of TIMP-1. Assuming a 1:1 stoichiometry (MMP-1 molecular weight of 55 kD and TIMP-1 molecular weight of 28 kD), a complex containing 3.2 ng of protein would contain approximately 2.1 ng of MMP-1. If we also assume that approximately 30% of the total MMP-1 was present in the active form (a conservative estimate based on the inset in Figure 2), essentially all of the active MMP-1 present in the culture fluid was likely bound. At the same time, most of the TIMP-1 was not bound to the enzyme.

Consistent with this, culture fluid from Omniscan-treated skin was found to have excess inhibitory activity. Our interpretation of these findings is that while both MMP-1 and TIMP-1 are elevated in organ culture fluid following GBCA stimulation, the level of TIMP-1 is sufficient to block collagenolytic activity that might otherwise prevent excess collagen deposition.

It should be noted that MMP-1 is not the only mammalian collagen-degrading enzyme. MMP-8 (neutrophil collagenase) and MMP-13 (collagenase-3) are also capable of cleaving intact fibrillar collagen (28,29). Since the tissue biopsies were obtained from normal healthy volunteers and exposed to Omniscan *in vitro*, MMP-8 would not be expected as this enzyme is a neutrophil product. While it is possible that MMP-13 might be present, we have been unable to detect significant amounts of this enzyme in organ-cultured skin (27). Irrespective of which enzyme(s) is/are actually responsible for collagen degradation in the skin, the important point is that collagenolytic enzyme activity is reduced to undetectable levels in skin exposed to chelated gadolinium compounds *ex vivo*.

Past studies have demonstrated coordinated alterations in MMP-1 and TIMP-1 in a number of different settings (30–34). Our own past studies have shown high levels of both enzyme and inhibitor in skin associated with basal cell tumors (23,24), and other studies have demonstrated increases of both in skin exposed to acute ultraviolet irradiation (25,27). At the molecular level, MMP-1 and TIMP-1 expression are regulated through several different signaling cascades. In general, mitogen-activated protein (MAP) kinase signaling is associated with increased MMP and TIMP expression (25,35,36). Signaling through the phosphatidylinositol-3 (PI3) kinase pathway further upregulates MMP-1; in contrast, PI3 kinase signaling has a dampening effect on TIMP-1 expression (37–39). Thus, depending on which of the two pathways is more highly activated, one might expect to see a relatively higher level of either MMP-1 or TIMP-1. Of interest in this regard, our recent study with human dermal fibroblasts (40) demonstrated that stimulation with Omniscan led to the same upregulation of MMP-1 and TIMP-1 as was observed in whole skin (18). A MAP kinase inhibitor (U0126) suppressed the upregulation of both enzyme and inhibitor, while a PI3 kinase inhibitor (LY294002) downregulated MMP-1 but further upregulated TIMP-1.

It is tempting to speculate that suppression of collagenolytic activity is directly responsible for excess matrix deposition in NSF skin lesions. This may be too simplistic, however. TIMP-1 may have functions other than simply altering extracellular matrix turnover. For example, MMP inhibitors help regulate the inflammatory response (41). Matrix deposition in NSF lesions may be due to subtle effects on inflammation rather than to a direct consequence of altered matrix turnover.

Additionally, MMP inhibitors stimulate proliferation in a number of different types of cells, including cells of mesenchymal origin (42,43). Consistent with this, the use of broad-spectrum synthetic MMP inhibitors in preclinical models of disease (e.g., cancer and arthritis) as well as in clinical studies has been shown to result in musculoskeletal complications characterized histologically by fibroplasia and clinically by joint stiffening (44–49). In light of these past findings, it would not be unreasonable to suggest a role for TIMP-1 in the fibroblast proliferation response to Omniscan and, perhaps, in the fibroplastic changes observed in NSF lesional skin. While possible, our own studies do not support this suggestion. Using culture conditions that have been found in past studies to support GBCA-induced fibroblast proliferation (18,40), we were unable to show a proliferative response to TIMP-1 over a wide range of concentrations (10–250 ng/mL) (Figure 5). This was in spite of the fact that when the cells were exposed to TIMP-1 (150 ng/mL) under identical conditions, increased deposition of type I collagen into the cell layer/substratum was observed. Our conclusion from these studies is that the strong induction of proliferation seen in Omniscan-treated fibroblasts (17,

18,40) is not a direct proliferative response to TIMP-1. More likely, the same increase in MAP kinase signaling that is responsible for TIMP-1 elevation underlies the increase in cell growth. Additional studies will be needed to address this issue.

Although our data suggest that TIMP-1 is responsible for inhibition of collagenolytic activity in GBCA-treated, organ-cultured skin, this may not be the only mechanism of enzyme inhibition. Our data also suggest a potential role for the metal ion chelators coupled to gadolinium in Omniscan and the other GBCAs that are currently in clinical use. MMP-1 and other metalloproteinases have a Zn^{2+} atom at the active site and depend on Ca^{2+} for their conformational state and catalytic activity (50). Metal ion chelators inhibit metalloproteinase function. When Omniscan was examined for direct inhibition of collagenolytic activity, concentrations of 500 μM and above were strongly inhibitory. While this may appear to be a high concentration, subjects in complete renal failure may have gadolinium blood levels that are higher than this after an MRI procedure (51). Whether metal ion chelation plays a significant role in collagenase suppression *in vivo* will require additional studies with other approaches than the ones used here. Our data simply suggest that a role is theoretically possible.

Interestingly, in regard to chelator effects, previous studies have suggested that differential stability among the various chelated gadolinium compounds contributes to the putative differences in capacity to induce NSF in susceptible individuals (52–54). Presumably, reactions occur in biological fluids that separate gadolinium from its chelator (transmetallation). While most studies have focused on the effects of “free” gadolinium, the separated chelators themselves might not be inert. Alternatively, separation of gadolinium from the chelator may not be required for enzyme inhibition by the chelator. Inhibition of collagenolytic activity was observed rapidly after addition of the GBCA to the activated enzyme.

In summary, while there is increased production of MMP-1 in human skin exposed to Omniscan, collagenolytic enzyme activity does not increase; it actually decreases. This is due, in part at least, to concomitant upregulation of TIMP-1. Additionally, the same GBCAs that upregulate TIMP-1 also have direct anti-collagenolytic activity, possibly due to the metal ion chelators that are present. Based on these findings, it is tempting to speculate that suppression of collagenolytic activity is directly responsible for excess matrix deposition in NSF skin lesions. It should be kept in mind, however, that regardless of what is observed using normal skin in an *ex vivo* model, there is no definitive evidence that these changes are related to NSF in any way. All we can say at present is that the findings are intriguing and, in our opinion, warrant further study.

Acknowledgments

This study was supported in part by grants GM 77724 and GM 80779 from the National Institutes of Health, Bethesda, MD.

REFERENCES

1. Cowper SE, Robin HS, Steinberg SM, et al. Scleromyxedema-like cutaneous diseases in renal-dialysis patients. *Lancet* 2000;356:1000–1001. [PubMed: 11041404]
2. Swartz RD, Crofford LJ, Phan SH, et al. Nephrogenic fibrosing dermopathy: a novel cutaneous fibrosing disorder in patients with renal failure. *Am J Med* 2003;114:563–572. [PubMed: 12753880]
3. Grobner T. Gadolinium--a specific trigger for the development of nephrogenic fibrosing dermopathy and nephrogenic systemic fibrosis? *Nephrol Dial Transpl* 2006;21:1104–1108.
4. Marckmann P, Skov L, Rossen K, et al. Nephrogenic systemic fibrosis: suspected causative role of gadodiamide used for contrast-enhanced magnetic resonance imaging. *J Am Soc Nephrol* 2006;17:2359–2362. [PubMed: 16885403]

5. Yerram P, Saab G, Karuparthi PR, et al. Nephrogenic systemic fibrosis: a mysterious disease in patients with renal failure-role of gadolinium-based contrast media in causation and the beneficial effect of intravenous sodium thiosulfate. *Clin J Am Soc Nephrol* 2007;2:258–263. [PubMed: 17699422]
6. Rydahl C, Thomsen HS, Marckmann P. High prevalence of nephrogenic systemic fibrosis in chronic renal failure patients exposed to gadodiamide, a gadolinium-containing magnetic resonance contrast agent. *Invest Radiol* 2008;43:141–144. [PubMed: 18197066]
7. Khurana A, Runge VM, Narayanan M, et al. Nephrogenic systemic fibrosis: a review of 6 cases temporally related to gadodiamide injection (omniscan). *Invest Radiol* 2007;42:139–145. [PubMed: 17220732]
8. Mendoza FA, Artlett CM, Sandorfi N, et al. Description of 12 cases of nephrogenic fibrosing dermatopathy and review of the literature. *Semin Arthritis Rheum* 2006;35:238–249. [PubMed: 16461069]
9. Wiginton CD, Kelly B, Oto A, et al. Gadolinium-based contrast exposure, nephrogenic systemic fibrosis, and gadolinium detection in tissue. *AJR Am J Roentgenol* 2008;190:1060–1068. [PubMed: 18356456]
10. Hope TA, Herfkens RJ, Denianke KS, et al. Nephrogenic systemic fibrosis in patients with chronic kidney disease who received gadopentetate dimeglumine. *Invest Radiol* 2009;44:135–139.
11. McNeill AM, Barr RJ. Scleromyxedema-like fibromucinosi in a patient undergoing hemodialysis. *Int J Derm* 2002;41:364–367. [PubMed: 12100695]
12. Neudecker BA, Stern R, Mark LA, et al. Scleromyxedema-like lesions of patients in renal failure contain hyaluronan: a possible pathophysiological mechanism. *J Cutan Pathol* 2005;32:612–615. [PubMed: 16176298]
13. Sieber MA, Pietsch H, Walter J, et al. A preclinical study to investigate the development of nephrogenic systemic fibrosis: a possible role for gadolinium-based contrast media. *Invest Radiol* 2008;43:65–75. [PubMed: 18097279]
14. Sieber MA, Lengsfeld P, Walter J, et al. Gadolinium-based contrast agents and their potential role in the pathogenesis of nephrogenic systemic fibrosis: the role of excess ligand. *J Magn Reson Imaging* 2008;27:955–962. [PubMed: 18425843]
15. Stenvinkel P. Inflammation in end-stage renal failure: could it be treated? *Nephrol Dial Transplant* 2002;17:33–38. [PubMed: 12147775]
16. Wermuth PJ, Del Galdo F, Jiménez SA. Induction of the expression of profibrotic cytokines and growth factors in normal human peripheral blood monocytes by gadolinium contrast agents. *Arthritis Rheum* 2009;60:1508–1518. [PubMed: 19404939]
17. Edward M, Quinn JA, Mukherjee S, et al. Gadodiamide contrast agent ‘activates’ fibroblasts: a possible cause of nephrogenic systemic fibrosis. *J Pathol* 2008;214:584–593. [PubMed: 18220317]
18. Varani J, DaSilva M, Warner RL, et al. Effects of gadolinium-based magnetic resonance imaging contrast agents on human skin in organ culture and human skin fibroblasts. *Invest Radiol* 2009;44:74–81. [PubMed: 19077912]
19. Varani J, Perone P, Fligiel SE, et al. Inhibition of type I procollagen production in photodamage: correlation between presence of high molecular weight collagen fragments and reduced procollagen synthesis. *J Invest Dermatol* 2002;119:122–129. [PubMed: 12164934]
20. Varani J, Fay K, Perone P. MDI-301, a non-irritating retinoid, induces changes in human skin that underlie repair. *Arch Dermatol Res* 2007;298:439–448. [PubMed: 17146625]
21. Lateef H, Stevens MJ, Varani J. All-trans-retinoic acid suppresses matrix metalloproteinase activity and increases collagen synthesis in diabetic human skin in organ culture. *Am J Pathol* 2004;165:167–174. [PubMed: 15215172]
22. Varani J, Perone P, Griffiths CE, et al. All-trans retinoic acid (RA) stimulates events in organ-cultured human skin that underlie repair. Adult skin from sun-protected and sun-exposed sites responds in an identical manner to RA while neonatal foreskin responds differently. *J Clin Invest* 1994;94:1747–1756. [PubMed: 7962521]
23. Varani J, Hattori Y, Chi Y, et al. Collagenolytic and gelatinolytic matrix metalloproteinases and their inhibitors in basal cell carcinoma of skin: comparison with normal skin. *Br J Cancer* 2000;82:657–665. [PubMed: 10682680]

24. Yucel T, Mutnal A, Fay K, et al. Matrix metalloproteinase expression in basal cell carcinoma: relationship between enzyme profile and collagen fragmentation pattern. *Exp Mol Pathol* 2005;79:151–160. [PubMed: 16004981]
25. Fisher GJ, Datta SC, Talwar HS, et al. Molecular basis of sun-induced premature skin ageing and retinoid antagonism. *Nature* 1996;379:335–339. [PubMed: 8552187]
26. Fisher GJ, Wang ZQ, Datta SC, et al. Pathophysiology of premature skin aging induced by ultraviolet light. *N Engl J Med* 1997;337:1419–1428. [PubMed: 9358139]
27. Brennan M, Bhatti H, Nerusu KC, et al. Matrix metalloproteinase-1 is the major collagenolytic enzyme responsible for collagen damage in UV-irradiated human skin. *Photochem Photobiol* 2003;78:43–48. [PubMed: 12929747]
28. Page-McCaw A, Ewald AJ, Werb Z. Matrix metalloproteinases and the regulation of tissue remodeling. *Nat Rev Mol Cell Biol* 2007;8:221–233. [PubMed: 17318226]
29. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* 2003;92:827–839. Review. [PubMed: 12730128]
30. Jung K, Nowak L, Lein M, et al. Matrix metalloproteinases 1 and 3, tissue inhibitor of metalloproteinase-1 and the complex of metalloproteinase-1/tissue inhibitor in plasma of patients with prostate cancer. *Int J Cancer* 1997;74:220–223. [PubMed: 9133459]
31. Keyszer G, Lambiri I, Keysser M, et al. Matrix metalloproteinases, but not cathepsins B, H, and L or their inhibitors in peripheral blood of patients with rheumatoid arthritis are potentially useful markers of disease activity. *Z Rheumatol* 1998;57:392–398. [PubMed: 10025098]
32. Kato S, Yasukawa H, Fujii T, et al. Coordinate regulation of matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 expression in human vascular smooth muscle cells. *Connect Tissue Res* 2000;41:143–153. [PubMed: 10992160]
33. Mizumoto H, Saito T, Ashihara K, et al. Expression of matrix metalloproteinases in ovarian endometriomas: immunohistochemical study and enzyme immunoassay. *Life Sci* 2002;71:259–273. [PubMed: 12034345]
34. Naveau A, Reinald N, Fournier B, et al. Gingival fibroblasts inhibit MMP-1 and MMP-3 activities in an ex-vivo artery model. *Connect Tissue Res* 2007;48:300–308. [PubMed: 18075816]
35. Barchowsky A, Frlleta D, Vincenti MP. Integration of the NF-kappaB and mitogen-activated protein kinase/AP-1 pathways at the collagenase-1 promoter: divergence of IL-1 and TNF-dependent signal transduction in rabbit primary synovial fibroblasts. *Cytokine* 2000;12:1469–1479. [PubMed: 11023661]
36. Zeigler ME, Chi Y, Schmidt T, et al. Role of ERK and JNK pathways in regulating cell motility and matrix metalloproteinase 9 production in growth factor-stimulated human epidermal keratinocytes. *J Cell Physiol* 1999;180:271–284. [PubMed: 10395297]
37. Lu Y, Wahl LM. Production of matrix metalloproteinase-9 by activated human monocytes involves a phosphatidylinositol-3 kinase/Akt/IKKalpha/NF-kappaB pathway. *J Leukoc Biol* 2005;78:259–265. [PubMed: 15800029]
38. Oh JH, Kim A, Park JM, et al. Ultraviolet B-induced matrix metalloproteinase-1 and -3 secretions are mediated via PTEN/Akt pathway in human dermal fibroblasts. *J Cell Physiol* 2006;209:775–785. [PubMed: 16972255]
39. Tong L, Smyth D, Kerr C, et al. Mitogen-activated protein kinases Erk1/2 and p38 are required for maximal regulation of TIMP-1 by oncostatin M in murine fibroblasts. *Cell Signal* 2004;16:1123–1132. [PubMed: 15240007]
40. Bhagavathula N, DaSilva M, Aslam MN, et al. Regulation of collagen turnover in human skin fibroblasts exposed to a gadolinium-based contrast agent. *Invest Radiol*. 2009 in press.
41. Cai G, Zhang X, Hong Q, et al. Tissue inhibitor of metalloproteinase-1 exacerbated renal interstitial fibrosis through enhancing inflammation. *Nephrol Dial Transplant* 2008;23:1861–1875. [PubMed: 18326884]
42. Hayakawa T, Yamashita K, Tanzawa K, et al. Growth-promoting activity of tissue inhibitor of metalloproteinase-1 (TIMP-1) for a wide range of cells. A possible new growth factor in serum. *FEBS Lett* 1992;298:29–32. [PubMed: 1544418]
43. Hayakawa T, Yamashita K, Ohuchi E, et al. Cell growth-promoting activity of tissue inhibitor of metalloproteinases-2 (TIMP-2). *J Cell Sci* 1994;107:2373–2379. [PubMed: 7844157]

44. Yoshiji H, Kuriyama S, Miyamoto Y, et al. Tissue inhibitor of metalloproteinases-1 promotes liver fibrosis development in a transgenic mouse model. *Hepatology* 2000;32:1248–1254. [PubMed: 11093731]
45. Yoshiji H, Kuriyama S, Yoshii J, et al. Tissue inhibitor of metalloproteinases-1 attenuates spontaneous liver fibrosis resolution in the transgenic mouse. *Hepatology* 2002;36:850–860. [PubMed: 12297832]
46. Zhang X, Chen X, Hong Q, et al. TIMP-1 promotes age-related renal fibrosis through upregulating ICAM-1 in human TIMP-1 transgenic mice. *J Gerontol A Biol Sci Med Sci* 2006;61:1130–1143. [PubMed: 17167153]
47. Hutchinson JW, Tierney GM, Parsons SL, et al. Dupuytren's disease and frozen shoulder induced by treatment with a matrix metalloproteinase inhibitor. *J Bone Joint Surg Br* 1998;80:907–908. [PubMed: 9768907]
48. King J, Zhao J, Clingan P, et al. Randomized double blind placebo control study of adjuvant treatment with the metalloproteinase inhibitor, Marimastat, in patients with inoperable colorectal hepatic metastases: significant survival advantage in patients with musculoskeletal side-effects. *Anticancer Res* 2003;23:639–645. [PubMed: 12680160]
49. Renkiewicz R, Qiu L, Lesch C, et al. Broad-spectrum matrix metalloproteinase inhibitor marimastat-induced musculoskeletal side effects in rats. *Arthritis Rheum* 2003;48:1742–1749. [PubMed: 12794843]
50. Iyer S, Wei S, Brew K, et al. Crystal structure of the catalytic domain of matrix metalloproteinase-1 in complex with the inhibitory domain of tissue inhibitor of metalloproteinase-1. *J Biol Chem* 2007;282:364–371. [PubMed: 17050530]
51. Grobner T, Prischl FC. Gadolinium and nephrogenic systemic fibrosis. *Kidney Int* 2007;72:260–264. [PubMed: 17507905]
52. Lin SP, Brown JJ. MR contrast agents: physical and pharmacologic basics. *J Magn Reson Imaging* 2007;25:884–899. [PubMed: 17457803]
53. Morcos SK. Extracellular gadolinium contrast agents: differences in stability. *Eur J Radiol* 2008;66:175–179. [PubMed: 18343072]
54. Frenzel T, Lengsfeld P, Schirmer H, et al. Stability of gadolinium-based magnetic resonance imaging contrast agents in human serum at 37 degrees C. *Invest Radiol* 2008;43:817–828. [PubMed: 19002053]

Figure 1. Collagen fragmentation by organ culture fluid from control and Omniscan-treated skin Polymerized type I collagen was exposed to organ culture fluid from untreated and Omniscan-treated (50 μ M) organ-cultured skin. SDS-PAGE analysis was used to resolve collagen fragments, which were then digitized and quantified. Values obtained with untreated (control) skin cultures were arbitrarily set to 1.0 and the Omniscan value compared to this. Values are means and standard errors based on tissue from 6 different subjects. Statistical significance of the data was determined using the Student t-test. *indicates statistically significant decrease compared to control at $p < 0.05$ level. **Inset:** The collagen alone lane shows a doublet corresponding to the α_1 and α_2 chains of intact type I collagen, but bands corresponding to the 1/4- and 3/4-sized fragments are not seen. Collagen exposed to culture fluid from a basal cell

carcinoma (BCC) demonstrates bands corresponding to the $\frac{1}{4}$ - and $\frac{3}{4}$ -sized fragments of type I collagen. Multiple additional fragments are seen as the $\frac{1}{4}$ - and $\frac{3}{4}$ -sized fragments are susceptible to further degradation. Collagen fragments are seen in the lane corresponding to collagen plus control skin culture fluid but are not seen in the lane corresponding to collagen plus culture fluid from Omniscan-treated skin.

Figure 2. MMP-1, TIMP-1, and MMP-1-TIMP-1 complexes in organ culture fluid from control and Omniscan-treated skin

Tissue was incubated for three days under control conditions or in the presence of Omniscan (50 μ M). At the end of the incubation period, culture fluid was collected and assayed as follows: **Upper panel.** MMP-1 assessed by western blotting (n=6). **Inset:** western blot from one subject. **Middle panel.** TIMP-1 assessed by ELISA (n=6). **Lower panel.** MMP-1 - TIMP-1 complex assessed by ELISA (n=5). Values shown are means and standard errors. Statistical significance of the data was determined using the Student t-test. *indicates statistically significant increase compared to control at p<0.05 level.

Figure 3. Inhibition of MMP-1-mediated collagen fragmentation by Omniscan

Polymerized type I collagen was exposed to purified, activated MMP-1 from synovial fibroblasts in the absence or presence of Omniscan (5–500 μ M). SDS-PAGE analysis was used to resolve collagen fragments, which were then digitized and quantified. Values obtained with MMP-1 alone were arbitrarily set to 1.0 and the other values compared to this. Values are means and standard deviations based on three separate experiments. Statistical significance of the data was determined by ANOVA, followed by paired-group comparisons. *indicates statistically significant decrease compared to control at $p < 0.05$ level. **Inset:** Collagen fragmentation pattern observed in one experiment. Lane 1 demonstrates intact collagen; lane 2 demonstrates the fragmentation pattern observed in the presence of activated MMP-1 alone; lanes 3–7 demonstrate fragmentation observed in the presence of activated MMP-1 and increasing concentrations (5–500 μ M) of Omniscan.

Figure 4. Inhibition of MMP-1-mediated collagen fragmentation by organ culture fluid from control and gadolinium chloride-treated skin

Polymerized type I collagen was exposed to purified, activated MMP-1 from synovial fibroblasts in the absence or presence of organ culture fluid from control and gadolinium chloride-treated (10–50 μ M) skin. SDS-PAGE analysis was used to resolve collagen fragments, which were then digitized and quantified. Values obtained with MMP-1 alone were arbitrarily set to 1.0 and the other values compared to this. Values are based on organ-cultured skin from one subjects. The experiment was repeated with skin from two subjects with similar results. **Inset:** Collagen fragmentation pattern observed with one subject. Lane 1 demonstrates intact collagen; lane 2 demonstrates the fragmentation pattern observed in the presence of

activated MMP-1 alone; lanes 3–6 demonstrate fragmentation observed in the presence of activated MMP-1 and culture fluids from tissue expose to 0, 10, 20 and 50 μ M gadolinium chloride.

Collagen Type 1

Figure 5. Proliferation and collagen deposition in human dermal fibroblasts exposed to exogenous TIMP-1

Proliferation: Proliferation was assessed under serum-free conditions in a growth factor-enriched medium as described in the Materials and Methods section. Values shown are means and standard deviations based on n=4 separate experiments. **Deposition of type I collagen.** Cells were incubated under control conditions or in the presence of exogenous TIMP-1 (150 ng/ml) for three days. At the end of the incubation period, lysates were prepared from the cell layer and assayed for type I collagen by western blotting. Bands were digitized and quantified. The value from the control was set to 1.0 and the value from TIMP-1 treatment compared to this. Values shown are means and standard deviations based on three separate experiments.

Statistical significance of the data was assessed using the Student t-test. *indicates statistically significant increase compared to control at $p < 0.05$ level. **Inset:** Intact $\alpha 1$ and $\alpha 2$ chains of type I collagen from one of the replicate experiments with β -tubulin (no change; not shown) used as control.