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Fibroblast Response to Gadolinium: Role for Platelet-Derived Growth Factor Receptor

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Abstract

Objective—The purpose of this study was to assess the effects of gadolinium (Gd^{3+}), provided as gadolinium chloride, on fibroblast function.

Materials and Methods—Human dermal fibroblasts in monolayer culture and intact skin in organ culture were exposed to the lanthanide metal (1–20 μM).

Results—Increased proliferation was observed, in association with up-regulation of matrix metalloproteinase-1 (MMP-1) and tissue inhibitor of metalloproteinases-1 (TIMP-1), without an apparent increase in production of type I procollagen. A PDGF receptor-blocking antibody inhibited fibroblast proliferation in response to Gd^{3+} as did inhibitors of signaling pathways – i.e., mitogen-activated protein (MAP) kinase and phosphatidylinositol-3 (PI3) kinase pathways – that are activated by PDGF.

Conclusion—The responses to gadolinium chloride are similar to responses previously seen with chelated Gd^{3+} in clinically-used MRI contrast agents. Fibroblast responses appear to reflect Gd^{3+} - induced PDGF receptor activation and down-stream signaling. Increased dermal fibroblast proliferation in conjunction with effects on MMP-1 and TIMP-1 could contribute to the fibroplastic / fibrotic changes seen in the lesional skin of individuals with nephrogenic systemic fibrosis.

Keywords

fibroblast; gadolinium; matrix metalloproteinase-1 (MMP-1); platelet-derived growth factor (PDGF-BB); tissue inhibitor of metalloproteinases-1 (TIMP-1)

INTRODUCTION

The lanthanide elements including lanthanum (Ln^{3+}) itself - which is technically not a lanthanide - constitutes a group of transition metals with atomic numbers between 57 and 71 in the atomic chart. These closely-related elements have an ionic radius and orbital configuration similar to that of Ca^{2+} , but a higher charge density (1). Lanthanide metals bind to Ca^{2+} - binding sites on proteins, and in some cases, the binding affinity is greater than that of Ca^{2+} , itself (2,3). Because of their high affinity for Ca^{2+} - binding sites on regulatory molecules, lanthanides have been used as probes for Ca^{2+} - mediated responses in epithelial cells (4).

Lanthanide metals also impact stromal cell function. Cerium (Ce^{3+}) has been shown to stimulate a proliferative response in fibroblasts from heart and lung (5,6). Proliferation is linked to oxidant-generation (5,6) and is associated with a diffuse cardiac fibrogenic response in experimental animals (7). Fibrogenic changes in the heart have been noted in individuals living in an area where Ce^{3+} levels are high in the soil (8,9). Two other lanthanides (Ln^{3+} and lutetium [Lu^{3+}]) have also been associated with stromal cell proliferation and fibrotic changes (10-12).

Recently, gadolinium (Gd^{3+}) has been linked to a fibrotic condition – i.e., nephrogenic systemic sclerosis (NSF) - in end-stage renal disease (ESRD) patients exposed to the transition metal during magnetic resonance imaging (MRI) (13-21). Studies from our laboratory have demonstrated that several of the gadolinium-based MRI contrast agents (GBCAs) are capable of stimulating a proliferative response in human dermal fibroblasts (22-24). Coupled with the proliferative response is up-regulation of matrix metalloproteinase-1 (MMP-1) and tissue inhibitor of metalloproteinases-1 (TIMP-1) without an apparent change in type I procollagen production. It is not clear whether these stimulatory effects on fibroblast function are responsible for the fibrotic changes in NSF or whether fibrosis might be a manifestation of toxicity, which is observed at high concentrations of Gd^{3+} (25,26). It is also not clear if the effects on fibroblast function are due to a unique response to chelated forms of the metal or whether salts of Gd^{3+} would produce similar responses. As part of our effort to elucidate the events that lead from Gd^{3+} exposure to fibrotic changes in the skin, we carried out a series of studies to assess a broad range of responses in human dermal fibroblasts exposed to gadolinium chloride, and to elucidate signaling pathways that lead from Gd^{3+} exposure to biological response.

MATERIALS AND METHODS

Reagents

Gadolinium chloride was obtained from Sigma Chemical Co and prepared as a 10 mM stock solution in distilled water. Omniscan (GE Healthcare) was obtained from In-Patient Pharmacy at the University of Michigan Hospitals. Omniscan is a sterile aqueous solution of gadodiamide (287 mg per mL [0.5M]) along with 12 mg per mL of sodium caldiumide (metal ion chelator), suitable for injection. Platelet-derived growth factor (PDGF-BB) and TGF- β were obtained from R&D Systems. A blocking antibody to the PDGF receptor was also obtained from R&D Systems (AB-20-NA). Antibodies recognizing the total and phosphorylated forms of the extracellular signal-related kinase (ERK) (antibodies 9102 and 9191) and antibodies recognizing the total and phosphorylated forms of AKT (antibodies 9272 and 9271) were obtained from Cell Signaling Technologies. Human synovial fibroblast-derived MMP-1 was from Chemicon. Antibodies recognizing human MMP-1 (AB6002) and human type I collagen (ab292) were obtained from Chemicon and Abcam respectively, and antibody against β -tubulin (SC-9104) was from Santa Cruz Biotechnology. The mitogen-activated protein (MAP) kinase inhibitor U0126 (agent 9903) and the phosphatidylinositol 3 (PI3) kinase inhibitor LY294002 (agent 9901) were from Cell Signaling Technologies.

Human subjects

Seven healthy subjects were recruited as skin donors. From each subject, six 2-mm, full-thickness punch biopsies of skin from the hip were obtained. The participation of human subjects in this study was approved by the University of Michigan Institutional Review Board and all subjects provided written informed consent prior to their inclusion in the study.

Human skin organ culture

Upon arrival in the laboratory, the punch biopsies were incubated in wells of a 24 well dish (one tissue piece per 0.5 mL of culture medium). Culture medium consisted of Keratinocyte Basal Medium (KBM) (Lonza). KBM is a low- Ca^{2+} (0.15 mM) modification of MCDB-153 medium. For our purposes, the culture medium was supplemented with calcium chloride to a final Ca^{2+} concentration of 1.55 mM. One well was left as a control while the others were treated with Gd^{3+} over a range of concentrations as indicated in the Results Section. Incubation was at 37°C in an atmosphere of 5% CO_2 and 95% air. Fresh culture medium was provided on days-2, -4 and -6. Organ culture-conditioned medium collected at day-2 was saved for assessment of type I procollagen, MMP-1 and TIMP-1 as described below. At the end of the incubation period (day-8), the tissue was fixed in 10% buffered formalin and embedded in paraffin. Three- μm thick sections were cut and stained with hematoxylin and eosin. Representative sections of each biopsy were selected for histological evaluation and photographed. The organ culture protocol used here is virtually identical to that described in several past reports (22,27-29).

Human dermal fibroblasts and epidermal keratinocytes in monolayer culture

Human foreskin tissue was obtained from circumcisions performed at the University of Michigan Hospitals. De-identified tissue samples were obtained under a waiver of IRB approval. This tissue was used to establish cultures of dermal fibroblasts and epidermal keratinocytes. Fibroblasts were grown using Dulbecco's Modified Minimal Essential Medium supplemented with nonessential amino acids and 10% fetal bovine serum (DMEM-FBS) as culture medium. Keratinocytes were grown in Keratinocyte Growth Medium (KGM) (Lonza). KGM contains the same basal medium as KBM but is supplemented with a mixture of growth factors including EGF, insulin, and bovine pituitary extract. Maintenance of both cell types was at 37°C in an atmosphere of 95% air and 5% CO_2 . Cells were used at passage 2-3. The isolation and handling of dermal fibroblasts and epidermal keratinocytes from skin samples has been described previously (27).

Proliferation

Fibroblasts were added to wells of a 24 well dish at 2×10^4 cells per well in DMEM-FBS, and allowed to attach. The wells were then washed two additional times with 1 mL of KGM supplemented with extracellular Ca^{2+} to a final concentration of 1.55 mM. Our past studies have demonstrated that when KGM is supplemented with extracellular Ca^{2+} to this final concentration, it provides a potent fibroblast growth medium (22). After washing, duplicate wells were counted to provide accurate "zero-time" values. One mL of Ca^{2+} - supplemented KGM was added to each remaining well. Gd^{3+} was added over a range of concentrations with or without additional agents as indicated in the Results Section. Incubation was for 3 days at 37°C in an atmosphere of 95% air and 5% CO_2 . At the end of the incubation period, culture fluids were harvested for assessment of MMP-1, TIMP-1, type I procollagen and type I collagen as described below. Cells were harvested with trypsin-EDTA and counted.

In some experiments, fibroblasts were added to wells of a 6-well dish at 3×10^5 cells per well. Cell attachment and growth was exactly as above. 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 a "lysis buffer" (Pierce Biotech) according to the manufacturer's instructions. Cell lysates were used to assess signaling intermediates and type I collagen (described below).

Proliferation studies with keratinocytes were carried out in a similar fashion, except that growth medium consisted of KGM with 0.15 mM Ca^{2+} (optimized for proliferation).

MMP-1

Western blotting with a rabbit polyclonal anti-MMP-1 antibody was used to assess MMP-1 levels (22). Briefly, samples consisting of conditioned-medium 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 desired antibody, diluted 1:1000 in 5% nonfat milk / TTBS. Thereafter, the membranes were washed with TTBS and bound antibody detected using SuperSignal WestPico Chemiluminescent Substrate (Thermo Scientific). Images were scanned, digitized and quantified. 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.

TIMP-1

Culture fluids were assayed for TIMP-1 by enzyme-linked immunosorbent assay (ELISA) using a commercially-available assay kit (R&D Systems) (22,24).

Type I procollagen

Culture fluids were assayed for type I procollagen by ELISA (Takara Bio, Inc.) as described previously (22). Type I procollagen contains the N- and C-terminal peptide sequences that are present at synthesis and cleaved during extracellular polymerization. Therefore, assessment of the precursor peptides provides a measure of newly synthesized collagen precursor.

Collagenolytic activity

Collagen degradation by enzymes present in fibroblast culture fluid was assessed as described previously (24). 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, Ca²⁺-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 fibroblast culture fluids or to human synovial fibroblast-derived MMP-1 as a positive control. Prior to testing, activation of the proenzyme was accomplished by exposure of the culture fluid or purified enzyme to 1 µg of crystalline trypsin for 5 minutes at 37°C following by 10 µg of SBTI. Intact collagen exposed to buffer alone served as a negative control. After incubation for 18 hours at 37°C, the supernatant fluids from control and treated collagen were removed, and collagen fragmentation was assessed. This was done by quantifying the ¼ and ¾ length collagen fragments released into the supernatant fluid after resolution by SDS-PAGE and staining with Coomassie brilliant blue.

Collagen deposition

To assess deposition of type I collagen into the cell layer, cell lysates were prepared using lysis buffer as described above. Total protein content of the cell lysates was determined and 20 µg of total protein was loaded and resolved using acrylamide gels. Gels were resolved in the presence of SDS and reducing buffer. Protein was transferred to nitrocellulose membranes and probed with a rabbit antibody to human type I collagen.

Cell signaling

The same lysates were used to assess signaling intermediates. Equal amounts of protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed for phospho- and total-ERK and phospho- and total-AKT.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by the Bonferroni post-test for selected pairs (GraphPad Prism version 4.00 for Windows, GraphPad Software). Data were considered significant at $p < 0.05$.

RESULTS

Gd³⁺ - induced responses in human dermal fibroblasts

Fibroblast responses to Gd³⁺ are presented in Figure 1. As can be seen from the upper panel, increased proliferation was observed at concentrations of 5 and 10 μM with the peak concentration at 10 μM . Cell numbers declined at concentrations above the optimal. Overt cytotoxicity was observed at 100 μM (not shown).

The same concentrations of Gd³⁺ that induced proliferation in dermal fibroblasts also stimulated increases in both MMP-1 and TIMP-1 (Figure 1; middle and lower panels). Since there were increases in both MMP-1 and TIMP-1, additional studies were carried out to assess collagenolytic activity in culture fluids from control and Gd³⁺ - treated cells. Collagenolytic activity – i.e., capacity to degrade intact polymerized type I collagen into $\frac{1}{4}$ and $\frac{3}{4}$ length fragments was virtually undetectable in control culture fluid and not elevated following Gd³⁺ stimulation (Figure 2, panels B and C). This is in spite of the fact that virtually all of the MMP-1 was converted to the active form by the trypsin / SBTI activation protocol (Figure 2, panel A).

Next, we assessed cell culture fluids from Gd³⁺ - treated fibroblasts for type I procollagen and for intact type I collagen. As seen in the upper panel of Figure 3, there was no stimulation of type I procollagen production. However, when intact collagen was assessed by Western blotting in the same culture fluids, the level was higher in culture fluid from cells exposed to Gd³⁺ than in control culture fluid (Figure 3; lower panel).

In a final set of studies, epidermal keratinocytes were also examined for response to Gd³⁺. Unlike fibroblasts, there was no increase in proliferation over the range of 1-100 μM . At the highest concentration, there was a fall-off in growth (not shown).

Organ culture studies: MMP-1, TIMP-1 and type I procollagen levels in Gd³⁺ - treated human skin organ culture fluid

Intact human skin from seven different subjects was exposed to Gd³⁺ in organ culture, and MMP-1, TIMP-1 and type I procollagen assessed. Consistent with results from fibroblasts in monolayer culture, increased levels of MMP-1 and TIMP-1 were observed in organ culture fluid following Gd³⁺ - exposure (5-20 μM) (Figure 4, upper-left and upper-right panels). Also consistent with monolayer culture results, there was no increase in the level of type I procollagen (Figure 4, lower-left panel). At the end of the 8-day incubation period, the tissue was fixed in 10% buffered formalin and evaluated histologically. There was no evidence of necrosis (Figure 4, lower-right panel).

Fibroblast responses to TGF- β and PDGF-BB: Comparison with Gd³⁺ - induced responses

Experiments were carried out to determine the signaling pathways that are activated by Gd³⁺ - exposure and lead to fibroblast responses. In the first studies, we compared Gd³⁺ to two known fibrogenic growth factors (TGF- β and PDGF-BB). TGF- β was examined at 1-10 ng/ml and PDGF-BB was used at 1-25 ng/ml. Data from these studies are summarized in Table 1. TGF- β treatment had no effect on fibroblast cell number, but as expected, led a large increase in type I procollagen production (7.4-fold over control). Additionally, MMP-1 was decreased by greater than 90% in response to TGF- β while there was little change in the

TIMP-1 level. These responses to TGF- β stimulation are consistent with findings that are well-established in the literature (30,31), but are unrelated to what was observed with Gd³⁺. In contrast, when fibroblasts were stimulated with PDGF-BB, the responses were virtually indistinguishable from those seen following Gd³⁺ exposure. That is, PDGF-BB stimulated fibroblast proliferation and increases in both TIMP-1 and MMP-1. There was little effect on type I procollagen (Table 1).

A blocking antibody to the PDGF receptor was tested for ability to interfere with Gd³⁺ - induced responses. Using fibroblast proliferation as the end-point, a concentration of antibody (10 μ g/ml) that inhibited PDGF-BB - induced growth by 47% was able to reduce proliferation in response to Gd³⁺ by 37% (Table 2). The same antibody had no effect on basal proliferation (i.e., in culture medium containing EGF, insulin and pituitary extract but without Gd³⁺ (Table 2).

In parallel studies, a preparation of chelated gadolinium (i.e., the MRI contrast agent, Omniscan) was assessed for ability to stimulate fibroblast proliferation under control conditions and in the presence of the same PDGF receptor-blocking antibody. Consistent with our previous reports (22-24), fibroblasts responded to Omniscan (25 μ M) with increased proliferation (Table 2). Similar to what was observed with Gd³⁺, the fibroblast proliferative response to Omniscan was suppressed by the antibody directed against the PDGF receptor (39% inhibition) (Table 2).

Intracellular signaling events in fibroblast response to Gd³⁺

ERK phosphorylation was assessed as an indicator of MAP kinase activity and AKT phosphorylation was assessed as an indicator of PI3 kinase activity. In parallel, we assessed the ability of MAP kinase and PI3 kinase antagonists to interfere with fibroblast responses to Gd³⁺. ERK phosphorylation in treated fibroblasts is presented in the upper panel of Figure 5. An increase in phosphorylation was observed as early as five minutes after exposure, and increased phosphorylation was also observed at 15 and 30 minutes. Longer-term studies indicated that increased ERK phosphorylation was still evident 48 hours post-treatment (not shown). There was, as expected, no significant change in total ERK expression over the same time period (Figure 5, upper panel).

The lower panel of Figure 5 demonstrates AKT phosphorylation in fibroblasts at 5, 15 and 30 minutes after exposure to Gd³⁺. A modest but consistent increase in AKT phosphorylation was apparent. Unlike what was observed with ERK, the phosphorylation pattern for AKT was not sustained in that beyond 30 minutes, there were no differences between control and treated cells (not shown). As expected, there was no change in total AKT protein at any time-point examined (Figure 5, lower panel).

In parallel studies, human dermal fibroblasts were exposed to Gd³⁺ in the presence of U0126 (10 μ M) or LY294002 (25 μ M). Effects on proliferation, MMP-1 and TIMP-1 were assessed. Figure 6 demonstrates that in the presence of U0126 (inhibitor of ERK activation), all three responses were reduced to near background levels (i.e., levels observed in the absence of stimulator and inhibitor) or slightly below. In the presence of the PI3 kinase inhibitor (LY294002), proliferation and MMP-1 were also inhibited. The MMP-1 level was substantially below background levels. The TIMP-1 level, however, was not reduced. Quite the opposite, the TIMP-1 level was substantially up-regulated over the level induced by Gd³⁺ alone (Figure 6).

DISCUSSION

Several issues are raised by these findings. The first is the potential mechanism(s) through which Gd^{3+} stimulates responses in fibroblasts. Because of its ionic size and orbital configuration, Gd^{3+} is a close mimic of Ca^{2+} , but with an overall higher charge-density (1). Gd^{3+} binds to Ca^{2+} -binding sites on proteins; in some cases, with higher affinity than that of Ca^{2+} itself (2,3). In epithelial cells, Gd^{3+} interacts with the extracellular calcium-sensing receptor (CaSR) to modulate Ca^{2+} -generated growth- and differentiation-regulating signals (4). While a CaSR-like protein has been identified on stromal cells (32), its role is not understood. The data presented here strongly suggest that in human dermal fibroblasts, Gd^{3+} (directly or indirectly) modulates signaling through the PDGF receptor. This is based on i) the similarity between fibroblast responses to Gd^{3+} and responses to PDGF-BB - similarities not seen with EGF or TGF- β , ii) similarities in the down-stream intracellular signaling cascades activated by Gd^{3+} (described here) and PDGF-BB (33), and iii) the strong suppression of Gd^{3+} -induced fibroblast proliferation with an antibody that targets the PDGF receptor. Also of interest in regard to the role of the PDGF receptor is the requirement for hyaluronan synthesis as an intermediary event in PDGF-induced proliferation (33). A recent study from our laboratory showed that hyaluronan levels are increased in GBCA-exposed fibroblasts (34).

Our data - both the information presented here, and the findings from our recent studies with chelated Gd^{3+} (22-24,34) - suggest that the major effects of fibroblast exposure to Gd^{3+} or Gd^{3+} -containing compounds is on proliferation and collagen degradation rather than on collagen production, *per se*. This is not proven, however. Failure to detect an increase in type I procollagen in the culture fluid may simply reflect the fact that most of the newly elaborated procollagen is directly deposited into the matrix (35), and that what is measured in solution is only residual material. Alternatively, there is no *a priori* reason to postulate increased procollagen synthesis. An increase in the number of collagen-producing cells coupled with decreased degradation could account for the increased collagen deposition noted here. Additionally, it should be noted that increased type I procollagen synthesis is related to TGF- β signaling. When TGF- β was included in the culture medium, significantly increased amounts of type I collagen were detected in the culture medium (Table 1). Two other points that may be of relevance to this discussion include the following: First, we showed in a past study (23) with one of the GBCAs (Omniscan) that there was no increase in type I procollagen mRNA by RT-PCR while mRNAs for both MMP-1 and TIMP-1 were elevated under the same conditions. Second, Gd^{3+} (and other lanthanoids) may have effects on collagen deposition that are independent of both synthesis and breakdown, *per se*. Specifically, it has been shown in the past that certain of the lanthanoid metals bind directly to the collagen molecule and promote fibrillogenesis (36,37). Additional studies will be required to completely assess all of the potential mechanisms through which these biologically-active metals can affect collagen metabolism and, ultimately, the amount deposited into the tissue.

A second issue is the relationship between the findings described here with Gd^{3+} (provided as gadolinium chloride) and previous results with the chelated forms of Gd^{3+} used in MRI contrast agents. Our own previous work (22,23,34) as well as work by others (38) has shown that a number of different GBCAs are capable of stimulating fibroblast proliferation directly. The same alterations in MMP-1 and TIMP-1 noted here are also observed following GBCA stimulation (22,23). It has been suggested that NSF reflects instability of the contrast agents and separation of Gd^{3+} from its chelator *in vivo*. This is based, in part, on dissociation kinetic and thermodynamic stability considerations (39,40), on results from experimental animal models (41-44) and on stability studies in human serum (45). The similarity between the findings made here with gadolinium chloride and the previous findings with chelated-

Gd^{3+} would seem to be supportive of this view. There are differences, however. With Omniscan, one of the GBCAs, fibroblast proliferation is induced at concentrations as low as 0.5 μM (22) (lower than that seen here with gadolinium chloride). At the upper end, GBCA-induced fibroblast proliferation can be seen at concentrations as high as 5 to 25 mM (or higher, depending on the agent) (22). With gadolinium chloride, in contrast, cytotoxicity is observed at concentrations of 50-100 μM (concentrations only 5 to 10-fold above those that optimally support fibroblast responses). Our tentative interpretation of these observations is that when Gd^{3+} is introduced into a biological solution as a simple salt, it rapidly attaches to available protein or to some other “carrier molecule” that allows it to remain soluble long enough to interact with potential target cells through moieties such as the PDGF receptor. If the concentration of Gd^{3+} is too high, the lanthanoid is cytotoxic (25,26). In addition, the excess metal forms insoluble precipitates with phosphate, hydroxide or other anions. It was shown in a past study that when gadolinium chloride was injected into rats, the metal accumulated in liver and spleen (46). This was presumed to reflect formation of insoluble complexes and phagocytosis by reticuloendothelial cells. It was shown in the same study that a chelated form of the metal was rapidly cleared through the kidneys and did not accumulate in liver or spleen. The question is not what can happen to Gd^{3+} when it comes in contact with biological materials, but what form of the molecule produces fibrogenic responses, what form is cytotoxic and what form may be simply inert. Addressing these questions is critical for understanding potential pathogenic mechanisms. While our data do not argue for or against de-chelation as a requirement for effects on fibroblast function as noted with the various GBCAs, these *in vitro* findings do suggest that if dechelation occurs, it does not rapidly produce large amounts of “free” Gd^{3+} (or one might expect to see toxicity and / or formation of insoluble precipitates as noted with excess gadolinium chloride).

What are the implication of these findings to NSF or, potentially, to other fibrotic conditions? While there is no direct evidence at this point to prove a connection between the *in vitro* findings and the pathological changes seen in NSF lesional skin, it is interesting to note that NSF is often referred to as a mucinous fibroplasia or as a scleromyxedema-type of lesion rather than as a true sclerotic disease (13,14,47). The fact that Gd^{3+} (introduced either as the simple salt or as a chelated moiety) directly stimulates fibroblast proliferation rather than procollagen production (22-24,34) is consistent with this. It is also interesting that two therapeutic agents (imatinib and rapamycin) with activity against the PDGF receptor itself, or with signaling intermediates that are down-stream of PDGF receptor activation have efficacy in NSF (48,49). Finally, a small molecule inhibitor with activity against the PDGF receptor has been shown to mitigate symptoms of fibrosis in gadodiamide-treated experimental animals (http://www.arraybiopharma.com/_documents/Publications/PubAttachment329.pdf). These data are consistent with a role for PDGF receptor activation in NSF, but additional studies will be needed before we can know for certain how important the PDGF signaling pathway is to NSF.

It is also tempting to speculate that these results may have implications beyond NSF. Patients in renal failure are susceptible to a number of inflammatory skin complications and many of these have a fibrosing component (50-53). The signaling pathways identified here, as well as the down-stream consequences – i.e, altered MMP-1 and TIMP-1 - may contribute to the pathophysiology of some of these conditions.

Finally, while our focus is on Gd^{3+} , members of the lanthanide family of transition elements are similar to one another in properties (1). Fibroproliferative / fibrotic changes have also been noted in experimental studies with some lanthanides other than Gd^{3+} . Among these are Ln^{3+} itself, as well as Ce^{3+} and Lu^{3+} (5-12). Ce^{3+} , in addition, has been linked to a diffuse cardiac fibroplastic condition seen among inhabitants of an area of India where the soil

concentration of this metal is high (8,9). Of particular interest, Pietsch et al. (54) demonstrated in a recent study that chelated forms of two different lanthanoids (i.e., europium and holmium) produced skin lesions in rats following five consecutive daily injections. The lesions induced by DTPA-europium were similar in intensity to those induced by chelated-Gd³⁺, while the lesions induced by holmium appeared to be milder. It would not be unreasonable to suggest that the fibrogenic changes observed with lanthanides other than Gd³⁺ reflect mechanisms similar to those operative with Gd³⁺. Most investigators assume that fibrotic changes reflect precipitation of the metal salt in the affected tissue, leading to a cytotoxic response, to activation of TGF- β / Smad signaling and increased collagen synthesis, or to phagocytosis and activation of the phagocytic cells (30,31,55-57). Our studies suggest that modulation of collagen breakdown may also be important. It should be noted that while our data have been generated with dermal fibroblasts, other investigators have suggested a role for circulating fibrocytes in NSF (58,59). The origin of the two populations may be different. However, when circulating fibrocytes enter the dermal tissue and differentiate into cells with collagen-metabolizing capacity, they respond to extracellular signals in a similar manner to that of resident fibroblasts (60). It seems reasonable to acknowledge at this point that multiple cell types and multiple mechanisms may contribute to the formation of fibrotic skin changes in NSF.

Adverse potential aside, it would be interesting to ask if one or more of the lanthanide metals (properly formulated and delivered) might be utilized for connective tissue repair. Increasing the production of collagen and decreasing its breakdown in atrophic skin is one strategy for preventing chronic ulcer formation in individuals who are at risk (i.e., aged individuals, people with diabetes or individuals on corticosteroids etc). Chronic ulcers occur with frequency and with devastating consequences in such individuals (61-63). The limited solubility of the inorganic lanthanide salts would almost certainly preclude using such salts in any topical preparation. On the other hand, organic complexes containing lanthanide metals are already available (64). While these are being developed, primarily, for systemic use, there is no *a priori* reason to not consider the use of such complexes in a topical preparation. Thus, while Gd³⁺ and other lanthanide metals can produce adverse effects at high concentrations in susceptible individuals, the use of inherently stable compounds, the expected low blood levels after topical application, and rapid clearance from the circulation in individuals with healthy kidney function should make topical use attractive as a novel approach to skin damage repair.

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References

1. Evans, CH., editor. Biochemistry of the Lanthanides. New York: Plenum Press; 1990.
2. Pidcock E, Moore GR. Structural characteristics of protein binding sites for calcium and lanthanide ions. J Biol Inorg Chem. 2001; 6:479–489. [PubMed: 11472012]
3. Huang Y, Zhou Y, Castiblanco A, et al. Multiple Ca²⁺ binding sites in the extracellular domain of the Ca²⁺ - sensing receptor corresponding to cooperative Ca²⁺ response. Biochemistry. 2009; 48:388–398. [PubMed: 19102677]

4. Kallay E, Bajna E, Wrba F, et al. Dietary calcium and growth modulation of human colon cancer cells; Role of the extracellular calcium-sensing receptor. *Cancer Detect Prevent.* 2000; 24:127–136. [PubMed: 10917132]
5. Preeta R, Nair RR. Stimulation of cardiac fibroblast proliferation by cerium: a superoxide anion-mediated response. *J Molec Cell Cardiol.* 1999; 31:1573–1580. [PubMed: 10423354]
6. Nair RR, Preeta R, Smith G, et al. Variation in mitogenic response of cardiac and pulmonary fibroblasts to cerium. *Biol Trace Element Res.* 2003; 94:237–246.
7. Kumar BP, Shivakumar K, Kartha CC, et al. Magnesium deficiency and cerium promote fibrogenesis in rat heart. *Bull Environ Contam Toxicol.* 1996; 57:517–524. [PubMed: 8694866]
8. Valiathan MS, Kartha CC, Eapen JT, et al. A geochemical basis for endomyocardial fibrosis. *Cardiovasc Res.* 1989; 23:647–648. [PubMed: 2598218]
9. Valiathan MS, Kartha CC. Endomyocardial fibrosis - the possible connection with myocardial levels of magnesium and cerium. *Int J Cardiol.* 1990; 28:1–5. [PubMed: 2194985]
10. Yu S, Hu J, Yang X, et al. La^{3+} - induced extracellular signal-regulated kinase (ERK) signaling via a metal-sensing mechanism linking proliferation and apoptosis in NIH 3T3 cells. *Biochemistry.* 2006; 45:11217–11223. [PubMed: 16964983]
11. Yu S, Hu J, Yang X, et al. Lanthanum induces extracellular signal-regulated kinase phosphorylation through different mechanisms in HeLa cells and NIH 3T3 cells. *BioMetals.* 2006; 19:13–18. [PubMed: 16502327]
12. Haley TJ, Komesu N, Efros M, et al. Pharmacology and toxicology of lutetium chloride. *J Pharm Sci.* 2006; 53:1186–1188. [PubMed: 14249433]
13. Cowper SE, Robin HS, Steinberg SM, et al. Scleromyxoedema-like cutaneous diseases in renal-dialysis patients. *Lancet.* 2000; 356:1000–1001. [PubMed: 11041404]
14. Swartz RD, Crofford LJ, Phan SH, et al. Nephrogenic fibrosing dermatopathy: a novel cutaneous fibrosing disorder in patients with renal failure. *Am J Med.* 2003; 114:563–572. [PubMed: 12753880]
15. Grobner T. Gadolinium--a specific trigger for the development of nephrogenic fibrosing dermatopathy and nephrogenic systemic fibrosis? *Nephrol Dial Transplant.* 2006; 21:1104–1108. [PubMed: 16431890]
16. 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]
17. 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]
18. Rydahl C, Thomsen HS, Marckmann R. 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]
19. Shabana WM, Cohan RH, Ellis JH, et al. Nephrogenic systemic fibrosis: a report of 29 cases. *Am J Radiol.* 2008; 190:736–741.
20. Cowper SE, Rabach M, Girardi M. Clinical and histological findings in nephrogenic systemic fibrosis. *Eur J Radiol.* 2008; 66:191–199. [PubMed: 18325705]
21. Hope TA, Herfkens RJ, Denianke KS, et al. Nephrogenic systemic fibrosis in patients with chronic kidney disease who received gadopentate dimeglumine. *Invest Radiol.* 2009; 44:135–139. [PubMed: 19151610]
22. 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 Radiology.* 2009; 44:74–81.
23. Bhagavathula N, DaSilva M, Aslam MN, et al. Regulation of collagen turn over in human skin fibroblasts exposed to a gadolinium-based contrast agent. *Invest Radiol.* 2009; 45:15–23.
24. Perone P, Weber S, DaSilva M, et al. Collagenase activity is suppressed by elevated tissue inhibitor of metalloproteinase-1 (TIMP-1) in human skin exposed to a gadolinium-based MRI contrast agent. *Invest Radiol.* 2010; 45:42–48. [PubMed: 19996758]

25. Spencer A, Wilson S, Harpur E. Gadolinium chloride toxicity in the mouse. *Hum Exp Toxicol*. 1998; 17:633–637. [PubMed: 9865421]
26. Kubota Y, Takahashi S, Takahashi I, et al. Different cytotoxic response to gadolinium between mouse and rat alveolar macrophages. *Toxicol In Vitro*. 2000; 14:309–319. [PubMed: 10906437]
27. Varani J, Perone P, Griffiths CEM, 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]
28. Perone P, Fay K, Varani J. MDI-301, a non-irritating retinoid, induces changes in human skin that underlie repair. *Arch Dermatol Res*. 2007; 298:439–448. [PubMed: 17146625]
29. 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]
30. Vanlaethem J. Transforming growth factor beta promotes development of fibrosis after repeated courses of acute pancreatitis in mice. *Gastroenterology*. 1996; 110:576–582. [PubMed: 8566606]
31. Cutroneo KR, White SL, Phan SH, Ehrlich HP. Therapies for bleomycin induced lung fibrosis through regulation of TGF- β 1 induced collagen gene expression. *J Cell Physiol*. 2007; 211:585–589. [PubMed: 17387717]
32. Dvorak MM, Riccardi D. Ca^{2+} as an extracellular signal in bone. *Cell Calcium*. 2004; 35:249–255. [PubMed: 15200148]
33. Li L, Asteriou T, Bernert B, et al. Growth factor regulation of hyaluronan synthesis and degradation in human dermal fibroblasts: importance of hyaluronan for the mitogenic response to PDGF-BB. *Biochem J*. 2007; 404:327–336. [PubMed: 17324121]
34. DaSilva M, O'Brien Deming M, Fligiel SEG, Dame MK, Johnson KJ, Swartz R, Varani J. Responses of human skin in organ culture to a gadolinium-based MRI contrast agent. Comparison of skin from patients in end-stage renal failure with skin from healthy subjects. *Invest Radiol*. 2010 in press.
35. Canty EG, Kadler KE. Commentary: Procollagen trafficking, processing and fibrillogenesis. *J Cell Sci*. 2005; 118:1341–1353. [PubMed: 15788652]
36. Evans CH, Drouven BJ. The promotion of collagen polymerization by lanthanide and calcium ions. *Biochem J*. 1983; 213:751–758. [PubMed: 6615457]
37. Drouven BJ, Evans CH. Collagen fibrillogenesis in the presence of lanthanides. *J Biol Chem*. 1986; 261:11792–11797. [PubMed: 3745166]
38. 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]
39. Morcos SK. Extracellular gadolinium contrast agents: differences in stability. *Eur J Radiol*. 2008; 66:175–179. [PubMed: 18343072]
40. Lin SP, Brown JJ. MR contrast agents: physical and pharmacological basics. *J Magn Reson Imaging*. 2007; 25:884–899. [PubMed: 17457803]
41. 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]
42. 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]
43. Sieber MA, Lengsfeld P, Frenzel T, et al. Preclinical investigation to compare different gadolinium-based contrast agents regarding their propensity to release gadolinium in vivo and to trigger nephrogenic systemic fibrosis-like lesions. *Eur Radiol*. 2008; 18:2164–2173. [PubMed: 18545998]
44. Pietsch H, Lengsfeld P, Steger-Hartmann T, et al. Impact of renal impairment on long-term retention of gadolinium in the rodent skin following the administration of gadolinium-based contrast agents. *Invest Radiol*. 2009; 44:226–233. [PubMed: 19252439]

45. Frenzel T, Lengsfeld P, Schirmer H, et al. Stability of gadolinium-based magnetic resonance imaging contrast agents in human serum at 37°C. *Invest Radiol.* 2008; 43:817–828. [PubMed: 19002053]
46. Barnhart JL, Kuhnert N, Bakan DA, Berk RN. Biodistribution of GdCl₃ and Gd-DTPA and their influence on proton magnetic relaxation in rat tissues. *Magnetic Res Imaging.* 1987; 5:221–231.
47. McNeill AM, Barr RJ. Scleromyxedema-like fibromucinosi in a patient undergoing hemodialysis. *Int J Derm.* 2002; 41:364–367. [PubMed: 12100695]
48. Kay J, High WA. Imatinib mesylate treatment of nephrogenic systemic fibrosis. *Arthritis & Rheum.* 2008; 58:2543–2548. [PubMed: 18668587]
49. Swaninathan S, Arbiser JL, Hiatt KM, et al. Rapid improvement of nephrogenic systemic fibrosis with rapamycin therapy: Possible role of phospho-70-ribosomal-s6 kinase. *J Am Acad Dermatol.* 2010; 62:343–345. [PubMed: 20115955]
50. Patterson JW. The perforating disorders. *J Am Acad Dermatol.* 1984; 10:561–581. [PubMed: 6371074]
51. Randle HW. Keratotic papules of chronic renal failure: the process of transepithelial elimination. *Arch Dermatol.* 1983; 119:874–875. [PubMed: 6639106]
52. Hood AF, Hardegen GL, Zarate AR, et al. Kyrle's disease in patients with chronic renal failure. *Arch Dermatol.* 1982; 118:85–88. [PubMed: 7059223]
53. Nickoloff BJ, Noodleman FR, Abel EA. Perforating pseudoxanthoma elasticum associated with chronic renal failure and hemodialysis. *Arch Dermatol.* 1985; 121:1321–1322. [PubMed: 4037828]
54. Pietsch H, Jost G, Frenzel T, Raschke M, Walter J, Schirmer H, Hutter J, Sieber MA. Efficacy and safety of lanthanoids as X-ray contrast agents. *Euro J Radiol.* 2010 Epub ahead of print.
55. Gong H. Uncommon causes of occupational interstitial lung diseases. *Current Opin Pulmonary Med.* 1996; 2:405–411.
56. Steger-Hartmann T, Raschke M, Riefke B, et al. The involvement of pro-inflammatory cytokines in nephrogenic systemic fibrosis – a mechanistic hypothesis based on preclinical results from a rat model treated with gadodiamide. *Exp Toxicol Pathol.* 2009; 61:537–542. [PubMed: 19131226]
57. 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]
58. Ortonne N, Lipsker D, Chantrel F, et al. Presence of CD45RO+ CD34+ cells with collagen synthesis activity in nephrogenic fibrosing dermatopathy: a new pathogenic hypothesis. *Brit J Dermatol.* 2004; 150:1050–1052. [PubMed: 15149539]
59. Bellini A, Mattoli S. The role of the fibrocyte, a bone marrow-derived mesenchymal progenitor, in reactive and reparative fibroses. *Lab Invest.* 2007; 87:858–870. [PubMed: 17607298]
60. Strieter RM, Keeley EC, Hughes MA, Burdick ME, Mehrad B. The role of circulating mesenchymal progenitor cells (fibrocytes) in the pathogenesis of pulmonary fibrosis. *J Leuk Biol.* 2009 Epub ahead of print.
61. Ashcroft GS, Mills SJ, Ashworth JJ. Ageing and wound healing. *Biogerontology.* 2002; 3:337–345.
62. Gosain A, DiPietro LA. Aging and wound healing. *World J Surg.* 2004; 28:321–326. [PubMed: 14961191]
63. Wicke C, Bachinger A, Coerper S, et al. Aging influences wound healing in patients with chronic lower extremity wounds treated in a specialized wound care center. *Wound Rep Reg.* 2009; 17:25–33.
64. Barta CA, Sachs-Barrable K, Jia J, et al. Lanthanide containing compounds for therapeutic care in bone resorption disorders. *Dalton Transact.* 2007; 43:5019–5030.

Figure 1. Responses of human dermal fibroblasts to Gd³⁺

Upper panel. Proliferation. **Middle panel.** MMP-1 assessed by western blotting. **Insert:** western blot from one experiment. **Lower panel.** TIMP-1 assessed by ELISA. Values shown are means and standard errors based on 5-7 experiments for each response. Statistical significance of the data was assessed by ANOVA, followed by paired-group comparisons. *indicates statistically significant increase compared to controls at p<0.05 level.

Figure 2. Collagen fragmentation by culture fluids from control and Gd^{3+} - treated fibroblasts

A. MMP-1 western blot of culture fluids from control and Gd^{3+} - treated fibroblasts prior to activation (lanes 3 and 5, respectively) and following activation (lanes 4 and 6, respectively). **B.** SDS-PAGE analysis of intact collagen and collagen fragmentation pattern obtained following exposure of intact type I collagen to: lane 1: control buffer alone; lane 2: MMP-1 purified from human synovial fibroblasts; lane 3: culture fluid from control fibroblasts without activation; lane 4: culture fluid from control fibroblasts following activation; lane 5: culture fluids from Gd^{3+} - treated fibroblasts without activation and lane 6: culture fluids from Gd^{3+} - treated fibroblasts following activation. **C.** Bands corresponding to the $1/4$ and $3/4$ length fragments were digitized and quantified. Values are results from a single experiments. The experiment was run three times with similar results.

Figure 3. Production of type I procollagen and deposition of type I collagen in the cell layer in Gd³⁺-treated cells

Upper panel: Cells were incubated under control conditions or in the presence of Gd³⁺ for three days. At the end of the incubation period, cell culture fluids were collected and assayed for type I procollagen by ELISA. Values shown are means and standard errors based on 5 separate experiments. **Lower panel:** Lysates were prepared from the cell layer and assayed for type I collagen by western blotting. Values are means and ranges based on duplicate experiments. The insert showing intact type I collagen is from one of the experiments with β -tubulin (no change) used as control.

Figure 4. Functional changes in organ-cultured human skin exposed to Gd³⁺

Tissue was incubated for two days under control conditions or in the presence of Gd³⁺. At the end of the incubation period, culture fluids were collected and assayed as follows:

Upper left panel: MMP-1 assessed by western blotting. **Insert:** western blot from one experiment. **Upper right panel:** TIMP-1 assessed by ELISA. **Lower left panel:** Type I procollagen assessed by ELISA. Values shown are means and standard errors based on n=7 subjects. Statistical significance of the data was determined by ANOVA, followed by paired-group comparisons. *indicates statistically significant increase compared to controls at p<0.05 level. **Lower right panel:** Tissue histology after 8 days in organ culture under control conditions or in the presence of 20 μM Gd³⁺.

Figure 5. Intracellular signaling in Gd^{3+} - treated fibroblasts

Upper panel: MAP kinase signaling: Cells were incubated under control conditions or in the presence of 10 μ M Gd^{3+} for the indicated times. At the end of the incubation period, cell lysates were prepared and assayed for phospho-ERK and total ERK protein by western blotting. Values shown are means and standard errors based on n=3 separate experiments. The insert shows phospho-ERK and total-ERK from one of the replicate experiments.

Lower panel: PI3 kinase signaling. Cells were incubated under control conditions or in the presence of 10 μ M Gd^{3+} for the indicated times. At the end of the incubation period, cell lysates were prepared and assayed for phospho-AKT and total AKT protein by western blotting. Values shown are means and standard errors based on n=3 separate experiments. The insert shows phospho-AKT and total-AKT from one of the replicate experiments. Statistical significance of the data was assessed by ANOVA, followed by paired-group comparisons. *indicates statistically significant increase compared to the respective control value at $p < 0.05$ level.

Figure 6. Effects of U0126 and LY294002 on proliferation, MMP-1 production and TIMP-1 production in Gd³⁺ - treated fibroblasts

Cells were incubated under control conditions or in the presence of 10 μM Gd³⁺ with or without U0126 or LY294002 for three days. At the end of the incubation period, culture fluid was collected and assayed as follows. **Upper panel.** Cell proliferation assessed as cell counts. **Middle panel.** MMP-1 assessed by western blotting. **Insert:** western blot from one experiment. **Lower panel.** TIMP-1 assessed by ELISA. Values shown are means and standard errors based on three separate experiments. Statistical significance of the data was assessed by ANOVA, followed by paired-group comparisons. *indicates statistically significant increase compared to control at p<0.05 level. **indicates statistically significant decrease compared to Gd³⁺ alone at p<0.05 level. ***indicates statistically significant increase compared to Gd³⁺ alone at p<0.05 level.

Table 1Responses of TGF- β - treated and PDGF-BB - treated fibroblasts.

Treatment group	Proliferation	type I procollagen	MMP-1	TIMP-1
Control	1.0	1.0	1.0	1.0
1 μ M TGF- β	1.2 $\pm$ 0.1	4.5 $\pm$ 1.6 *	0.3 $\pm$ 0.1 **	1.0 $\pm$ 0.1
2 μ M TGF- β	1.3 $\pm$ 0.3	7.4 $\pm$ 0.9 *	0.3 $\pm$ 0.2	1.3 $\pm$ 0.2
5 μ M TGF- β	1.3 $\pm$ 0.2	3.9 $\pm$ 2.1 *	0.3 $\pm$ 0.1 **	1.2 $\pm$ 0.2
10 μ M TGF- β	1.0 $\pm$ 0.1	2.2 $\pm$ 0.7 *	0.1 $\pm$ 0.1 **	1.4 $\pm$ 0.4
1 μ M PDGF-BB	1.4 $\pm$ 0.3	0.9 $\pm$ 0.3	0.9 $\pm$ 0.2	1.1 $\pm$ 0.2
5 μ M PDGF-BB	1.6 $\pm$ 0.1 *	1.3 $\pm$ 0.4	10.0 $\pm$ 1.9 *	1.5 $\pm$ 0.4
10 μ M PDGF-BB	2.7 $\pm$ 0.1 *	1.1 $\pm$ 0.2	22.5 $\pm$ 7.5 *	2.2 $\pm$ 0.7 *
25 μ M PDGF-BB	2.4 $\pm$ 0.2 *	1.0 $\pm$ 0.3	30.0 $\pm$ 5.5 *	2.6 $\pm$ 0.4 *

Fibroblasts were incubated for 3 days in growth factor (EGF, pituitary extract and insulin) – enriched, serum-free culture medium and treated with TGF- β or PDGF-BB. Proliferation data are based on cell counts at the end of the 3-day incubation period. MMP-1 data are based on digitization of bands in Western blots. TIMP-1 data are based on ELISA. Control values were set at 1.0 and other values normalized to the control value. Values are means and standard errors from three separate experiments for TGF- β and five separate experiments for PDGF-BB. Statistical significance of the data was assessed by ANOVA, followed by paired-group comparisons.

* indicates statistically significant increase compared to control at $p < 0.05$ level.

** indicates statistically significant decrease compared to control at $p < 0.05$ level.

Table 2

Effects of a PDGF receptor-blocking antibody on proliferation of human dermal fibroblasts in response to Gd^{3+} and Omniscan.

Treatment groups	number of cells $\times 10^4$	Percent Inhibition
Control	2.1 ± 0.3	
+ control antibody (10 $\mu\text{g/ml}$)	2.0 ± 0.2	
+ anti-PDGFr antibody (10 $\mu\text{g/ml}$)	2.0 ± 0.3	
PDGF (10 μM)	$5.7 \pm 0.4^*$	
+ control antibody (10 $\mu\text{g/ml}$)	NA	
+ anti-PDGFr antibody (10 $\mu\text{g/ml}$)	$4.0 \pm 0.3^{**}$	47%
Gd^{3+} (10 μM)	$4.3 \pm 0.3^*$	
+ control antibody (10 $\mu\text{g/ml}$)	4.2 ± 0.2	
+ anti-PDGFr antibody (10 $\mu\text{g/ml}$)	$3.5 \pm 0.2^{**}$	37%
Omniscan (25 μM)	$3.9 \pm 0.3^*$	
+ control antibody (10 $\mu\text{g/ml}$)	3.8 ± 0.2	
+ anti-PDGFr antibody (10 $\mu\text{g/ml}$)	$3.2 \pm 0.2^{**}$	39%

Values shown are means and standard errors based on six separate experiments for control, Gd^{3+} and PDGF, and on four experiments with Omniscan. Statistical significance of the data was assessed by ANOVA, followed by paired-group comparisons.

* indicates statistically significant increase compared to negative control at $p < 0.05$ level.

** indicates statistically significant decrease compared to stimulating agent alone.

Percent inhibition values were calculated by subtracting the control value (2.1) from each value and determining residual growth of the stimulating agent plus PDGF-blocking antibody compared to growth in the presence of the stimulating agent alone.