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Responses of Human Skin in Organ Culture and Human Skin Fibroblasts to a Gadolinium-Based MRI Contrast Agent: Comparison of Skin from Patients with End-Stage Renal Disease and Skin from Healthy Subjects

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Abstract

Objective—Nephrogenic systemic fibrosis (NSF) is a clinical syndrome occurring in a small subset of patients with end stage renal disease (ESRD). Exposure to certain of the gadolinium-based contrast agents (GBCAs) during magnetic resonance imaging appears to be a trigger. The pathogenesis of the disease is largely unknown. The present study addresses potential pathophysiological mechanisms.

Materials and Methods—Here we have compared responses in organ-cultured skin and skin fibroblasts from individuals with ESRD to responses of healthy control subjects to Omniscan treatment.

Results—Treatment of skin from ESRD patients with Omniscan stimulated production of matrix metalloproteinase-1 (MMP-1) and tissue inhibitor of metalloproteinases-1 (TIMP-1), but not type I procollagen. The same treatment also stimulated an increase in hyaluronan production. Similar results were seen with skin from normal controls but basal levels were higher in ESRD patients. Fibroblasts in monolayer culture gave the same responses but there were no differences based on whether the cells were isolated from the skin of healthy subjects or those with ESRD.

Conclusion—These data indicate that Omniscan exposure alters an enzyme / inhibitor system responsible for regulating collagen turnover in the skin and directly stimulates hyaluronan production. The higher basal levels of type I procollagen, MMP-1, TIMP-1 and hyaluronan in the skin from ESRD patients could contribute to the sensitivity of this patient population to fibrotic changes which might be induced by exposure to some of the GBCAs.

Keywords

gadolinium-based contrast agent (GBCA); hyaluronan; matrix metalloproteinase I (MMP-1); nephrogenic systemic fibrosis (NSF); Omniscan; tissue inhibitor of metalloproteinases-1 (TIMP-1); type I procollagen

INTRODUCTION

Nephrogenic systemic fibrosis (NSF) is a systemic fibrotic disorder occurring in a small number of patients with reduced renal function (1-13). Exposure to certain of the gadolinium-based contrast agents (GBCAs), mostly for magnetic resonance imaging (MRI), may be a trigger of the disease (3,4,6,7-9,13). Histopathological features characteristic of NSF include the presence of dense, thickened collagen bundles in lesional skin. Strong staining with Alcian blue is also seen, suggesting elevated deposition of sulfated glycosaminoglycans and hyaluronan (14,15). Numerous, “plump” fibroblast-like cells are commonly seen in lesional skin. Some studies have reported only a minor increase in the number of proliferating interstitial cells, while others have reported a florid fibroplasia, resembling the cellular phase of acute wound healing (5). Although the majority of cases of the disease have been reported in patients with end-state renal disease (ESRD), it is not known if this simply reflects decreased GBCA clearance by individuals with kidney disease or whether patients in renal failure have additional perturbations that promote fibroplasia and fibrosis in the right circumstances. Pro-inflammatory and pro-fibrotic changes in ESRD are common (16-20).

In recent studies we used human skin in organ culture as a model system in which to assess the effects of several clinically-used GBCAs on skin structure / function (21). These studies showed that although there were differences in dose-response (Omniscan < Magnevist and Multihance < Prohance), all four of the GBCAs increased the levels of matrix metalloproteinase-1 (MMP-1; collagenase-1) and tissue inhibitor of metalloproteinases-1 (TIMP-1) without stimulating production of type I procollagen. Subsequent studies with Omniscan (22,23) demonstrated that the level of TIMP-1 in treated skin was sufficiently high that virtually all of the active MMP-1 was complexed with the inhibitor. Not surprisingly, collagenolytic activity was undetectable in the treated skin cultures, and collagen deposition increased.

Our previous studies were carried out using organ-cultured skin from healthy, adult subjects. Whether skin from ESRD patients would respond to Omniscan stimulation in a similar manner to that of skin from healthy subjects is not known. To begin addressing this issue, we obtained skin biopsies from a series of patients with ESRD and compared responses in organ culture to the responses in similarly-treated skin from a group of healthy control subjects. The results of this study are the subject of the present report.

MATERIALS AND METHODS

Reagents

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] with respect to gadolinium) along with 12 mg per ml of sodium caldiumide (metal ion chelator), suitable for injection. 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 subjects

Thirteen subjects with ESRD were recruited for this study. This included 6 females and 7 males with an age range from 25 to 80 years. The underlying causes of renal failure in the majority of subjects in this small cohort were hypertension (six) and diabetes (three). Interstitial nephritis, acute tubular necrosis and lupus were contributing factors in the other

subjects. All of the subjects were on hemodialysis and the duration of dialysis ranged from 1 to 30 years. All of the subjects had received erythropoietin during the course of treatment and some had been treated with iron as well. Two-mm full-thickness punch biopsies (6 per subject) were obtained from the hip. None of the 13 subjects had a history of prior exposure to any of the clinically used GBCAs. Ten healthy subjects (7 females and 3 males with an age range of 20 to 50 years) were also recruited to provide punch biopsies of hip skin. 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 500 μ L of culture medium). Culture medium consisted of Keratinocyte Basal Medium (KBM) (Lonza). KBM is a low- Ca^{2+} (0.15 mM) modification of Molecular, Cellular and Developmental Biology Medium 153 (MCDB-153). 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 Omniscan at 250 μ M. Incubation was at 37°C in an atmosphere of 5% CO_2 and 95% air. Fresh culture medium was provided at day-3 and day-6. Organ culture-conditioned medium collected at day-3 was saved for assessment of type I procollagen, MMP-1, TIMP-1 and hyaluronan as described below. At the end of the incubation period (day-6), the tissue was fixed in 10% buffered formalin and embedded in paraffin. Three- μ m thick sections were cut and stained with hematoxylin and eosin. Representative sections of each biopsy were selected for histological evaluation and photographed. Other sections (5- μ m) were stained with Alcian blue for detection of hyaluronan. Human skin in organ culture has been extensively used in the past in our laboratory. The organ culture protocol used here is virtually identical to that described in several past reports (21,23-25).

Human dermal fibroblasts in monolayer culture

Human dermal fibroblasts were isolated from 2-mm punch biopsies of skin as described previously (24). 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. Fibroblasts were maintained at 37°C in an atmosphere of 95% air and 5% CO_2 . Cells were used at up to passage 7.

Proliferation

Nine fibroblast isolates from six healthy subjects and three isolates from three different ESRD subjects were assessed for proliferation. Cells grown in 75-cm² flasks were harvested by brief (2 minute) exposure to a solution of 0.05% trypsin with 0.2 mg/mL ethylenediaminetetraacetic acid [EDTA]). The cells were added to wells of a 24 well dish at 3×10^4 cells per well in DMEM-FBS and allowed to attach and spread. The cells were then washed two additional times and incubated with serum-free, Ca^{2+} - supplemented Keratinocyte Growth Medium (KGM) (Lonza). KGM consists of the same basal medium as KBM but is supplemented with a mixture of growth factors including EGF, insulin, and bovine pituitary extract. Duplicate wells were counted to provide accurate “zero-time” values. The rest of the wells were used in the experiment. Duplicate wells served as control. Omniscan was added to the remainder of the wells over the concentration range of 0 to 250 μ M. 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 collected for assessment of hyaluronan as described below. Cells were harvested with trypsin-EDTA and counted.

Type I procollagen

Organ culture fluids from eight ESRD subjects were assayed for type I procollagen by enzyme-linked immunosorbent assay (ELISA) (Takara Bio. Inc.) as described previously (25). Type I procollagen contains the N- and C-terminal peptide sequences that are present at synthesis and, therefore, provides a measure of newly synthesized collagen precursor.

MMP-1

Organ culture fluid from 10 healthy control subjects and 13 ESRD patients were examined for MMP-1 and TIMP-1 production. Western blotting with a rabbit polyclonal anti-MMP-1 antibody (Chemicon, Temecula, CA) was used to assess MMP-1 levels (21,22). Briefly, organ culture and cell culture fluid 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) for one hour at room temperature, membranes were incubated overnight at 4°C with the desired 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 (Thermo Fisher Scientific Inc.). Images were scanned, digitized and quantified (Unscan-it, Silk Scientific Corp.). Western blots with purified human MMP-1 from human rheumatoid synovial fibroblasts were run in parallel in order to generate a standard curve. Values from organ culture fluids were obtained by comparison with the standard curve.

TIMP-1

Organ culture fluid from 10 healthy control subjects and 13 ESRD patients were assayed for TIMP-1 by ELISA using a commercially-available assay kit (R&D Systems) (25).

Hyaluronan

Hyaluronan was assessed in two ways. A commercial ELISA (R&D Systems) was used to quantify immunoreactive material in organ culture fluid from 8 normal subjects and 11 patients with ESRD and cell culture fluids from nine normal skin isolates and three isolates from patients with ESRD. Additionally, Alcian blue staining of histological sections (four normal and four ESRD skin samples) and fibroblasts in monolayer culture was used to obtain a measure of hyaluronan in the tissue matrix and cells (26). Alcian blue was obtained from Rowley Biochemical Inc. Since Alcian blue stains various complex carbohydrates, untreated slides and slides that had been exposed for one hour to hyaluronidase (500 µg/mL; Rowley Biochemical Inc.) were stained and evaluated in parallel.

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) or by the paired T-test where appropriate. 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 higher than the respective control values.

RESULTS

Effects of Omniscan on MMP-1 and TIMP-1 in organ culture fluid

In the first series of experiments, replicate 2-mm punch biopsies of sun-protected hip skin were obtained from a group of healthy donors and from patients with ESRD. Tissue was incubated in organ culture under control conditions or in the presence of Omniscan (250 µM with respect to gadolinium). Organ culture fluid collected at day-3 from control and Omniscan-treated skin was examined for levels of MMP-1 and TIMP-1. Results obtained

with 10 healthy subjects and 13 renal failure patients are summarized in Figure 1. It can be seen that with skin from normal healthy adults, Omniscan treatment resulted in increases in both MMP-1 and TIMP-1 levels (3.3-fold and 1.4-fold respectively, compared to control). This is similar to what we have reported previously (21,23). It can also be seen from the Figure that increases in MMP-1 and TIMP-1 were also observed in organ culture fluid from ESRD patients' skin (1.6-fold and 1.4-fold, respectively, compared to control). The major difference between the two groups of subjects was in basal levels. With both MMP-1 and TIMP-1, basal levels were higher in skin culture fluids from ESRD patients than in culture fluids from healthy control subjects. With MMP-1, the basal level was 3.2-fold higher in ESRD than in control, and with TIMP-1, the basal level was 1.6-fold higher in ESRD than in control.

In addition to quantifying MMP-1 and TIMP-1 in the organ culture fluids, we also assessed type I procollagen in the skin organ culture fluid from eight ESRD patients. Consistent with our previous report with skin from a cohort of 12 healthy subjects (21), Omniscan treatment did not increase type I procollagen levels in culture fluid from this group of subjects. However, when the basal level of type I procollagen in skin culture fluid from the ESRD patients (assessed here) was compared with the level in skin organ culture fluid from healthy adult subjects (our previous study) (21), the level was higher in the patients with ESRD (180 ± 66 ng per mL) than in normal healthy subjects (125 ± 19 ng/mL) (44% increase; $p < 0.05$ by unpaired T-test).

Hyaluronan production in control and Omniscan-treated skin from healthy subjects and patients with ESRD

Organ culture fluid from 8 healthy subjects and 11 ESRD patients was examined for hyaluronan by ELISA. Control culture fluid and culture fluid from Omniscan (250 μ M) - treated skin were compared. As was observed with the other moieties, basal hyaluronan levels were increased in ESRD as compared to control (Figure 2). Increased amounts were seen in both populations following Omniscan treatment (Figure 2). The relative increase was similar (50% increase in controls vs 67% increase in ESRD isolates).

In addition to assessing hyaluronan levels in organ culture fluid by ELISA, we also stained tissue sections from control and Omniscan-treated skin with Alcian blue. For this, tissue from four healthy control subjects and four ESRD patients was used. Duplicate tissue sections were stained for each condition. One of the duplicate sections was maintained as a control and the other section treated for one hour with 500 μ g/mL hyaluronidase prior to staining. Histological features from one ESRD patient are shown in the lower panel of Figure 2. In the control section (a), the connective tissue matrix had a pale blue color. When the tissue was exposed to hyaluronidase prior to staining (b), the pale blue color was lost. The same staining pattern was observed in sections from the Omniscan-treated tissue as seen in the control, but the staining of the matrix was more intense (c). Most of the staining was lost in the tissue section that had been pretreated with hyaluronidase prior to staining (d). The findings presented here were consistent with all 8 subjects. Staining was generally more intense with the ESRD patients than with healthy controls.

Effects of Omniscan on dermal fibroblasts from ESRD patients and healthy control subjects

Previous studies have demonstrated that dermal fibroblasts in monolayer culture respond to Omniscan stimulation with increased proliferation (21,22,27). Given the findings with organ-cultured skin from the two groups of subjects presented above, studies were carried out to determine if dermal fibroblasts from the same subjects would show similar basal differences as seen in organ-cultured skin, and if comparable responses to Omniscan would

be seen. Nine different isolates from six healthy skin donors and three isolates from three ESRD patients were used. Each isolate was treated with Omniscan over a wide range of concentrations and examined for proliferation as described in the Materials and Methods Section. The results from these studies are presented in Figure 3, where it can be seen that basal proliferation rates (i.e., in the absence of Omniscan) were similar, as were responses to Omniscan. The increase in response to Omniscan was 2.2-fold for control isolates and 2.1-fold for isolates from ESRD patients

The same fibroblast isolates were assessed for hyaluronan production under control conditions and following Omniscan (250 μ M) treatment. For this, cell culture fluids were collected at the end of the incubation period and assessed using the same assay procedure as described for organ culture fluids. Similar basal levels were seen in fibroblasts from both sources (1900 and 1800 ng per ml for control and ESRD isolates, respectively) and an increase in response to Omniscan was observed in both (21% increase in control isolates versus 50% increase in ESRD isolates (Figure 4).

DISCUSSION

NSF is a fibroproliferative / fibrotic condition that has been seen in a small subset of patients with ESRD (1-13). Why the disease is exclusively seen in this patient population is not completely understood. Failure to clear the chelated gadolinium compounds from the circulation is presumed to be at least partly responsible. While the GBCA half-life in individuals with healthy renal function is approximately 90 minutes, half-life in ESRD patients may be 50 hours or longer (28-31). Gadolinium has been detected in the tissues of several ESRD patients, in some individuals as long as two years after receiving a contrast-enhanced MRI (32-34). Data from animal models is supportive. Pietsch et al. (35) recently demonstrated that gadolinium could be detected in skin of partially-nephrectomized rats several months post injection. Although inability to clear the metal from the circulation is, undoubtedly, critical, this does not, in and of itself, explain the patho-physiology.

Past studies by a number of investigators have addressed mechanistic events that could play a role in the disease. Wermuth et al (36) recently showed that both chelated and unchelated gadolinium stimulated human blood monocytes to elaborate a number of pro-inflammatory and pro-fibrotic cytokines. Steger-Hartmann et al. (37), demonstrated elaboration of a number of pro-inflammatory cytokines in rats following exposure to Omniscan. The activation of inflammatory cells could explain at least some of the pathological features (excoriation) observed in animals exposed to gadolinium-containing compounds (38-41), as well as certain of the histopathological features (e.g., redness, induration, edema) seen in lesional skin in patients with NSF (1,2,11,12). Equally important, inflammatory cell activation could also be expected to influence the behavior of other cell types (i.e., those involved in collagen elaboration) in the developing NSF lesions. In particular, circulating fibrocytes have been postulated to play a role in NSF (42,43), presumably recruited in response to specific chemokines generated by activated inflammatory cells (37). Likewise changes in matrix production and breakdown by recruited fibrocytes as well as resident fibroblasts could be secondary to monocytes and other inflammatory cells reacting to the GBCA. Our own past studies have demonstrated that while there is no increase in type I procollagen production in organ-cultured human skin exposed to Omniscan, one of the major enzyme / enzyme inhibitor systems that regulate collagen turnover in the skin is affected. While our past data suggest that MMP-1 and TIMP-1 up-regulation reflects, in part at least, a direct response of resident fibroblasts to Omniscan (21,22), enzyme and inhibitor up-regulation could also be a response to pro-inflammatory cytokines and the down-stream signaling pathways activated (see below).

Regardless of whether the evolving NSF lesion reflects, primarily, direct or indirect responses in matrix-elaborating cells to gadolinium-containing compounds, a critical issue is whether there is something “unique” in patients with ESRD as compared to healthy individuals (over and above the failure to clear the contrast agents from the circulation) that could enhance a fibrotic response under a given set of conditions. As noted above (16-20), a chronic inflammatory state is common in patients with ESRD, so it would not be unreasonable to suggest an exaggerated response might occur in this population. Past epidemiological studies have provided evidence that beyond ESRD, *per se*, inflammatory changes in the vasculature as well as traumatic vascular injury, edema, infection, treatment with agents that have a pro-inflammatory potential (e.g., erythropoietin and iron) may be contributing factors to the development of NSF lesions in the small subset of ESRD patients that develop the disease (see 44-47 for recent reviews). Studies in experimental animals are supportive. For example, treatment of rats with high doses of erythropoietin and iron along with gadodiamide produced worse skin lesions than treatment with gadodiamide alone (48).

The current data provide further evidence that patients in ESRD have underlying perturbations that may permit an enhanced fibrotic response. To summarize our findings, levels of MMP-1 and TIMP-1 were elevated in response to Omniscan exposure in organ-cultured skin from both healthy subjects and individuals with ESRD. While type I procollagen levels did not increase in the skin of either population in response to Omniscan, another matrix component found in NSF lesional skin (e.g., hyaluronan) was elevated in both populations. The major difference was in baseline values (i.e., in the absence of Omniscan exposure), where levels of all four of these moieties were elevated as compared to what was seen in skin from healthy control subjects. Thus, the milieu in which the eliciting agent is presented may already be primed for a response.

What are the signaling pathways that modulate these responses in fibroblasts? Past studies have shown that MMP-1 and TIMP-1 production in fibroblasts is regulated through a number of intracellular signaling pathways including the mitogen-activated protein (MAP) kinase, nuclear factor-kappa B (Nf- κ B) and the phosphatidyl inositol 3 (PI3) kinase pathways (49-58). Hyaluronan synthesis is also influenced by the MAP kinase signaling (both p38 and ERK contributing) (56). Since these same signaling pathways are stimulated by a number of pro-inflammatory cytokines, proinflammatory mediators elaborated in renal failure (16,17,57,58) may contribute to the increased background levels of enzyme and inhibitor as well as increased hyaluronan in these patients. As noted above, studies by Wermuth et al. (36) and by Steger-Hartmann et al. (37) have demonstrated production of pro-inflammatory cytokines in response to GBCA treatment. Perhaps, production of pro-inflammatory cytokines by GBCA-activated inflammatory cells in the context of an already elevated background level is sufficient to convert sub-clinical elevations of MMP-1 / TIMP-1 into levels that are clinically-relevant. While findings with isolated fibroblasts - both in the present study and in past studies (21,22,27) - argue that stromal fibroblasts in the skin are, themselves, a direct target of GBCA action, there is no *a priori* reason to assume that these cells are the only target. Fibroblasts could be both a direct and indirect target of the agents.

It is tempting to speculate that the findings presented here and in our recent reports (21-23) help understand the patho-physiology of NSF. It must be kept in mind, however, that at present there is no evidence that they do. Additional work will be required to make the connection between what we observe *ex vivo* and what occurs in ESRD patients exposed to chelated-gadolinium compounds during MRI. It is also tempting to speculate that our 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 (59-62). Alterations in an enzyme / inhibitor system responsible for matrix turnover in the skin, as

well as alterations in the level of at least one matrix component may contribute to the pathophysiology of some of these conditions.

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Figure 1.

MMP-1 and TIMP-1 in human skin exposed to Omniscan. Biopsies were incubated for three days under control conditions or in the presence of Omniscan (250 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 and compared to a standard curve generated with human synovial fibroblast MMP-1. **Inset:** Western blot from one normal subject and one ESRD patient. **Lower panel.** TIMP-1 assessed by ELISA. Values shown are means and standard errors. Data are based on n=10 healthy control subjects and n=13 ESRD patients. Significance of the data was determined by ANOVA, followed by paired-group comparisons. *indicates statistically significant increase compared to healthy subjects at p<0.05 level; ** indicates statistically significant increase compared to the respective, untreated control at p<0.05 level.

Figure 2.

Upper panel: Hyaluronan in human skin exposed to Omniscan. Biopsies were incubated for three days under control conditions or in the presence of Omniscan (250 μ M). At the end of the incubation period, culture fluid was collected and assayed for hyaluronan by ELISA. Values shown are means and standard errors, based on n=8 healthy normal subjects and n=11 ESRD patients. Statistical significance of the data was determined by ANOVA, followed by paired-group comparisons. *indicates statistically significant increase compared to healthy control subjects at p<0.05 level; ** indicates statistically significant increase compared to respective, untreated control at p<0.05 level. **Lower panels:** Immunostaining of organ cultured skin for hyaluronan expression. Five- μ m sections were exposed to buffer alone or to hyaluronidase and then stained with Alcian blue. **A and B:** Organ-cultured skin from a patient with ESRD incubated without Omniscan (without and with hyaluronidase,

respectively). **C and D:** Organ-cultured skin from an ESRD patient incubated with 250 μ M Omniscan (without and with hyaluronidase, respectively).

Figure 3.

Effects of Omniscan on proliferation of dermal fibroblasts from healthy control subjects and patients with ESRD. Values are means and standard errors based on 9 isolates from 6 healthy subjects and 3 isolates from three ESRD patients. Statistical significance of the data at each concentration was assessed using the paired T-test. *indicates statistically significant increase compared to control at $p < 0.05$ level.

Figure 4. Hyaluronan levels in cell culture fluid assessed by ELISA. Values are means and standard errors based on 9 isolates from 6 healthy subjects and 3 isolates from three ESRD patients. 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.