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Gadolinium Compounds Signaling through TLR 4 and TLR 7 in Normal Human Macrophages: Establishment of a Proinflammatory Phenotype and Implications for the Pathogenesis of Nephrogenic Systemic Fibrosis

Peter J. Wermuth and Sergio A. Jimenez

Nephrogenic systemic fibrosis (NSF) is a generalized fibrotic disease occurring in some individuals with renal insufficiency following exposure to gadolinium-based contrast agents (GdBCA). Previous studies demonstrated that the GdBCA Omniscan upregulated several innate immunity pathways in normal differentiated human macrophages, induced rapid nuclear localization of the transcription factor NF-κB, and increased the expression and production of numerous profibrotic/proinflammatory cytokines, chemokines, and growth factors. To further examine GdBCA stimulation of the innate immune system, cultured human embryonic kidney 293 cells expressing one of seven different human TLRs or one of two human nucleotide-binding oligomerization domain-like receptors were exposed in vitro for 24 h to various GdBCA. The signaling activity of each compound was evaluated by its ability to activate an NF-κB–inducible reporter gene. Omniscan and gadodiamide induced strong TLR4- and TLR7-mediated reporter gene activation. The other Gd compounds examined failed to induce reporter gene activation. TLR pathway inhibition using chloroquine or an inhibitor of IL-1R–associated kinases 1 and 4 in normal differentiated human macrophages abrogated Omniscan-induced gene expression. Omniscan and gadodiamide signaling via TLRs 4 and 7 resulted in increased production and expression of numerous profibrotic/proinflammatory cytokines, chemokines, and growth factors, including CXCL10, CCL2, CCL8, CXCL12, IL-4, IL-6, TGF-β, and vascular endothelial growth factor. These observations suggest that TLR activation by environmental stimuli may participate in the pathogenesis of nephrogenic systemic fibrosis and of other fibrotic disorders including systemic sclerosis. The Journal of Immunology, 2012, 189: 318–327.

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The mechanisms underlying GdBCA stimulation of tissue fibrosis remain largely undetermined. GdBCA contain a single Gd³⁺ ion complexed to either a linear or a macrocyclic chelate that increases Gd³⁺ solubility and decreases Gd³⁺ toxicity (13–15). According to U.S. Food and Drug Administration data, all NSF cases in which the GdBCA used was identified have been associated with a linear GdBCA (16), most commonly Omniscan (62%) and Magnevist (32%). Other published reports raised the proportion of Omniscan-associated NSF cases to as high as 90% (17). A hypothesis to explain these observations suggested that less thermodynamically stable linear chelates undergo transmetallation, a process in which endogenous circulating ions such as Zn²⁺ displace Gd³⁺ at greater rates compared with macrocyclic chelates. It was further suggested that transmetallation is exacerbated by the markedly reduced GdBCA clearance rates in patients with renal insufficiency. However, in vitro experiments have demonstrated that exposure to both linear and macrocyclic GdBCA induces potent functional effects on cultured human normal dermal fibroblasts (8, 18–26), normal human PBMCs (27) and differentiated macrophages (28), and fibrocytes (29). One of these studies demonstrated strong activation of the NF-κB pathway in normal human macrophages following exposure to Omniscan and suggested that TLR activation mediated by the chelated Gd³⁺ compounds may be responsible for these effects (28).

Recently, the insight that TLRs are capable of recognition of a variety of endogenous damage-associated molecular patterns released from injured and inflamed tissues (30) has led to a pro-
posed role of innate immunity in the development of various fibrotic disorders including SSC and pulmonary fibrosis (31–39). Indeed, SSC patients display high levels of expression of IFN-responsive genes, markers of innate immune activation, which correlates with the modified Rodnan skin score, a measure of the degree of SSC skin involvement, and SSC sera induce IFN-α expression in normal PBMCs (34, 35, 40). Furthermore, the TLR3 ligand polyinosinic-polycytidylic acid induces marked activation of IFN-α and increased production of the potent profibrotic cytokine TGF-β in normal and SSC fibroblasts, resulting in autocrine stimulation of IFN-α- and TGF-β-responsive gene expression and dermal inflammation and fibrosis in mice (35). Affected tissues from SSC patients often exhibit chronic inflammation, suggesting that the release of endogenous TLR ligands during inflammation and TLR signaling may represent one mechanism that initiates and drives this autoimmune fibrotic disease (38). Interestingly, NSF occurs almost exclusively in patients with renal insufficiency (1–4), a clinical condition often accompanied by chronic microinflammation (41).

We have previously described changes in the transcriptome of normal differentiated human macrophages induced by exposure to the GdBCA Omniscan (28), including the upregulated expression of IFN-responsive genes accompanied by a rapid and intense NF-κB activation, as well as NF-κB–dependent expression of numerous proinflammatory/profibrotic cytokines, chemokines, and growth factors. The results of these studies have led to the hypothesis that GdBCA-induced TLR signaling activates expression of proinflammatory/profibrotic molecules (28). To test this hypothesis, we evaluated the effect of Omniscan on NF-κB activation in cells overexpressing a single TLR or nucleotide-binding oligomerization domain (NLR)–like receptor (NLR). We report that cells expressing TLR4 or TLR7 were responsive to Omniscan exposure as measured by the activation of a reporter gene under NF-κB control. We then compared the ability of various GdBCA to signal through TLR4 or TLR7 and examined the effect of two TLR pathway signaling inhibitors on Omniscan-mediated increases in proinflammatory/profibrotic cytokine, chemokine, and growth factor expression in normal human differentiated macrophages. The data presented in this study strongly indicate a role for TLR signaling by Omniscan in normal differentiated human macrophages, a pathway that may play a crucial role in the pathogenesis of NSF. These results also provide support to the participation of TLR in the pathogenesis of SSC and other inflammatory fibrotic disorders.

Materials and Methods

**Gd compounds**

Dotarem (Guerbet, Bloomington, IL), MultiHance (Braaco Diagnostics, Milan, Italy), ProHance (Braaco Diagnostics), and OptiMark (Mallinkrodt/Coviden, Hazelwood, IN) were supplied as sterile, aqueous solutions containing 500 mM Gd chelate. Omniscan and gadodiamide (provided by GE Healthcare, Chalfont St. Giles, U.K.) were supplied as sterile, aqueous solutions containing 287 mg/ml (500 mM) gadodiamide. The Omniscan solution contained an additional 12 mg/ml (25 mM) caldiamide sodium in water. Caldiamide (GE Healthcare) was supplied as a sterile aqueous solution containing 12 mg/ml (25 mM) caldiamide sodium in water. Gd-EDTA (GE Healthcare) was supplied as a sterile aqueous solution containing 250 mM Gd-EDTA in water. Gd-citrate (250 mM) was prepared by mixing 1 ml 250 μM Gd chloride and 1 ml 500 μM sodium citrate solutions at pH 7.4 (42). Gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA; Sigma-Aldrich, St. Louis, MO) was supplied as a sterile aqueous solution at pH 7.4. As positive controls, the cellular response to known specific TLR ligands containing Quanti-Blue (InvivoGen), to allow colorimetric detection of SEAP. As negative controls, the response to either 5, 10, or 25 ng/ml E. coli flagellin (TLR5), 1 μg/ml CpG oligodeoxynucleotide 2006, 100 ng/ml C12-iEDAP (NOD1), or 10 ng/ml L18-MDP (NOD2). Gd-EDTA (GE Healthcare) was supplied as a sterile aqueous solution containing 250 mM Gd-EDTA in water. Gd-citrate (250 mM) contained 250 mM Gd-EDTA. Gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA; Sigma-Aldrich, St. Louis, MO), ProHance (Omniscan), or gadodiamide; or to 2.7 μM Gd-EDTA; or to 0.25 mM of the chelate molecule caldiamide in 200 μl medium containing Quanti-Blue (InvivoGen) to allow colorimetric detection of SEAP.

**TLR screening**

Human embryonic kidney 293 (HEK293) cells were engineered to express different human TLRs (TLRs 2, 3, 4, 5, 7, 8, or 9) or two human NLRs (NOD1 or NOD2) were obtained from InvivoGen (San Diego, CA). For experiments the cells were seeded into 96-well plates at a density of 20,000 cells/well in DMEM containing 1% FBS (Life Technologies, Grand Island, NY), 1% vitamins, 2 mM glutamine, antibiotics, and fungizone (DMEM complete medium). These cells also express a reporter construct under the control of the NF-κB–inducible promoter resulting in expression of secreted alkaline phosphatase (SEAP) into the culture medium reflecting the levels of NF-κB activation. Cells in DMEM complete medium were exposed in duplicate for 20 h to 5 mM of one of the following Gd compounds: ProHance, Omniscan, or gadodiamide; or to 2.7 μM Gd-EDTA; or to 0.25 mM of the chelate molecule caldiamide in 200 μl medium containing Quanti-Blue (InvivoGen) to allow colorimetric detection of SEAP.

For TLR ligand dose–response experiments, the above procedure was performed using HEK293 cells expressing either human TLR4 or TLR7 cultured in DMEM complete medium at a density of 50,000 cells per well. The cells were exposed to 0.1, 0.5, or 5 mM of one of the following GdBCA: Gd-DTPA, Dotarem, MultiHance, ProHance, OptiMark, gadodiamide, or Omniscan; or to 2.7, 27, or 270 nM Gd-EDTA; or to 0.025, 0.05, or 0.25 mM of the chelate molecule caldiamide in 200 μl medium containing Quanti-Blue as described above. As positive controls, the response to 1.5, or 10 ng/ml E. coli K12 LPS (TLR4) or 1, 5, or 10 μg/ml gadigiquimod (TLR7) was measured. No significant effect on cell numbers or cytotoxicity was observed as examined by the WST-1 assay (Roche Diagnostics, Indianapolis, IN).

**Macrophage isolation and differentiation**

Normal human peripheral blood leukodetection filters were obtained from the Thomas Jefferson University Hospital Blood Bank following Institutional Review Board approval. Human PBMCs were isolated from the leukodetection filters by Ficoll-Hypaque gradient centrifugation (Amer- sham Pharmacia Biotech, Piscataway, NJ) and enriched for monocytes by adherence to plastic culture dishes for 2 h in RPMI 1640 containing 10% FBS (Hyclone Technologies), as described previously (27). To obtain terminally differentiated macrophages the macrophages were cultured in RPMI 1640 complete medium with 60 ng/ml M-CSF (BioVision, Mountain View, CA) and 25 ng/ml IL-10 (BioVision) for 7 d as described (28). Differentiated macrophages (5 × 105 cells/ml) were exposed for 24 h to 1 mM of one of the following GdBCA: Gd-DTPA, Dotarem, MultiHance, ProHance, OptiMark, Omniscan, or gadodiamide; or to 0.05 mM caldiamide; or to 0.27 mM Gd-EDTA or Gd-citrate. No significant effect on cell numbers or cytotoxicity was observed as examined by the WST-1 assay (Roche Diagnostics).

**TLR inhibition studies**

To confirm a role of TLRs in GdBCA stimulation of macrophages, normal human differentiated macrophages were washed with PBS and exposed to either 50 μM TLR inhibitor chloroquine (InvivoGen) or 300 nM IL-1R–associated kinase (IRAK)1/4 inhibitor 1-(2-(4-morpholinylethyl)-2-(3-nitrobenzoylamino)benzimidazole, N-(2-morpholinylethyl)-2-(3-nitrobenzoylamino)benzimidazole (Sigma-Aldrich) in RPMI 1640 complete medium for 1 h. This incubation was followed by addition of either PBS or PBS with 1 mM of either Omniscan, gadodiamide, or ProHance; or 0.05 mM caldiamide; or 2.7 μM Gd-EDTA for 24 h. As positive controls for TLR-dependent and TLR-independent macrophage stimulation, 100 ng/ml LPS (InvivoGen) or 10 μg/ml TNF-α (Pierce Biotechnology, Woburn, MA), respectively, were used. Macrophage samples cultured with an equal volume of PBS served as negative controls. No significant effect on cell numbers or cytotoxicity was observed on exposure to either 0.5 M NaCl or 100% ethanol for 30 min. After the WST-1 assay (Roche Diagnostics), Macrophage culture supernatants were isolated, filtered, and maintained frozen for subsequent studies. Cells were washed twice with PBS and then processed for RNA extraction using the RNeasy kit (Qiagen, Valencia, CA) according to the protocol recommended by the manufacturer as described previously (27).
Results

Identification of TLRs involved in Omniscan activation of NF-κB in HEK293 cells

To explore the ability of GdBCA to induce NF-κB activation via TLR and NLR signaling, HEK293 cells expressing one of seven human TLRs (TLRs 2, 3, 4, 5, 7, 8, or 9) or one of two human NLRs (NOD1 or NOD2) were exposed in DMEM complete medium for 24 h to one of five compounds: 5 mM linear GdBCA Omniscan; 5 mM gadodiamide, the linear Gd chelate component of Omniscan; 0.25 mM caldiamide, which is the amount of excess chelate present in the Omniscan formulation; 5 mM macrocyclic GdBCA ProHance; or to 2.7 μM nonchelated Gd compound Od-EDTA. The effect of these compounds on TLR or NLR signaling was assessed colorimetrically by measuring production of secreted alkaline phosphatase resulting from the activation of NF-κB in these cells. Because production of alkaline phosphatase is under the control of a stably integrated NF-κB–inducible promoter, the amount of alkaline phosphatase released by the cells is a direct reflection of the level of NF-κB activation. All agents tested were verified to be endotoxin free by the manufacturer and confirmed using a Luminus amebocyte lysate gel formation assay. No significant effect on cell numbers or cytotoxicity was observed as examined by the WST-1 assay (Roche Diagnostics).

Expression levels of IL-4, IL-6, IL-13, IFN-γ, TGF-β, VEGF, TLR4, TLR7, CCL2, CCL8, CXCL1, CXCL10, and CXCL11 in macrophage culture supernatants from the TLR inhibition studies described above

To identify the TLRs involved in Omniscan activation of NF-κB in HEK293 cells, expression level of IL-4, IL-6, IL-13, IFN-γ, TGF-β, VEGF, TLR4, TLR7, CCL2, CCL8, CXCL1, CXCL10, and CXCL11 in macrophage culture supernatants from the TLR inhibition studies described above

RNA interference

DharmaFECT 1 small interfering RNA (siRNA) transfection reagent, siGENOME SMARTPool siRNAs specific for human TLR4 and TLR7, and siGENOME RISC-free control siRNAs were purchased from Dharmacon (Lafayette, CO). Normal human macrophages (5 × 10⁶) differentiated as described above were plated in six-well plates and transfected with 100 nM siRNA for TLR4 or TLR7 or TLR4 plus TLR7 or with control (scrambled) siRNAs using DharmaFECT 1 (3 μl/well) according to the manufacturer’s instructions. After 24 h, macrophages were treated with each of 1 mM Omniscan, 1 mM gadodiamide, or 1 mM ProHance for 24 h or left untreated. Total RNA was extracted and RNA levels were assessed by real-time PCR as described above. No significant effect on cell numbers or cytotoxicity was observed as examined by the WST-1 assay (Roche Diagnostics).

Real-time PCR validation

Expression levels of IL-4, IL-6, IL-13, IFN-γ, TGF-β, VEGF, TLR4, TLR7, CCL2, CCL8, CXCL1, CXCL10, and CXCL11 (ITAC), and CXCL11 (ITAC) were assayed by real-time quantitative PCR utilizing SYBR Green chemistry (Applied Biosystems, Foster City, CA) following instructions. After 24 h, macrophages were treated with either 1 mM Omniscan, 1 mM gadodiamide, or 1 mM ProHance for 24 h or left untreated. Total RNA was extracted and RNA levels were assessed by real-time PCR as described above. No significant effect on cell numbers or cytotoxicity was observed as examined by the WST-1 assay (Roche Diagnostics).

Expression levels of IL-4, IL-6, IL-13, IFN-γ, TGF-β, VEGF, TLR4, TLR7, CCL2, CCL8, CXCL1, CXCL10, and CXCL11 in macrophage culture supernatants from the TLR inhibition studies described above

As portrayed in Fig. 1, 5 mM Omniscan induced significantly increased NF-κB–dependent SEAP production in HEK293 cells expressing either TLR4 or TLR7 compared with control cells incubated with endotoxin-free PBS. Omniscan did not cause statistically significant stimulation of reporter gene expression in the HEK293 cells expressing any of the other TLRs or NLRs. TLR7 expression cells showed a 5-fold increase in SEAP levels compared with the other NLRs in response to Omniscan. These changes in SEAP expression were observed for all replicates for TLR4- and TLR7-expressing cells. Gadodiamide, the Gd chelate in Omniscan, also induced increased NF-κB–dependent SEAP production in HEK293 cells to nearly identical to those induced in response to Omniscan. In contrast to the Gd chelate Omniscan, the macrocyclic Gd

Real-time PCR

Real-time PCR values reflect the mean and SD of three separate experiments each performed in triplicate with each of the three samples of normal human macrophages. The statistical significance of the real-time PCR data was assessed by a Student two-tailed t test. A p value < 0.05 was considered statistically significant.

Multiplex ELISA

SearchLight proteome array analyses (Aushon Biotechnology, Woburn, MA) were conducted to measure the levels of IL-4, IL-6, IL-13, IFN-γ, TGF-β, VEGF, TLR4, TLR7, CCL2, CCL8, CXCL1, CXCL10, and CXCL11 in macrophage culture supernatants from the TLR inhibition studies described above following procedures described previously (43). Briefly, culture supernatant samples were diluted 1:2, 1:50, or 1:1000 and incubated for 1 h on array plates that had been prespotted with capture Abs specific for each protein. Plates were decanted and washed three times with PBS before addition of a mixture of biotinylated detection Abs to each well. Following incubation with detection Abs for 30 min, plates were washed three times and incubated for 30 min with streptavidin-HRP. Plates were again washed, and SuperSignal Femto chemiluminescent substrate (Pierce Biotechnology) was added. The plates were immediately imaged using the SearchLight imaging system and data were analyzed using ArrayVision software (GE Healthcare).

Statistical analysis

Real-time PCR values reflect the mean and SD of three separate experiments each performed in triplicate with each of the three samples of normal human macrophages. The statistical significance of the real-time PCR data was assessed by a Student two-tailed t test. A p value < 0.05 was considered statistically significant.

To explore the ability of GdBCA to induce NF-κB activation via TLR and NLR signaling, HEK293 cells expressing one of seven human TLRs (TLRs 2, 3, 4, 5, 7, 8, or 9) or one of two human NLRs (NOD1 or NOD2) were exposed in DMEM complete medium for 24 h to one of five compounds: 5 mM linear GdBCA Omniscan; 5 mM gadodiamide, the linear Gd chelate component of Omniscan; 0.25 mM caldiamide, which is the amount of excess chelate present in the Omniscan formulation; 5 mM macrocyclic GdBCA ProHance; or to 2.7 μM nonchelated Gd compound Od-EDTA. The effect of these compounds on TLR or NLR signaling was assessed colorimetrically by measuring production of secreted alkaline phosphatase resulting from the activation of NF-κB in these cells. Because production of alkaline phosphatase is under the control of the level of NF-κB activation. All agents tested were verified to be endotoxin free by the manufacturer and confirmed using a Luminus amebocyte lysate gel formation assay. No significant effect on cell numbers or cytotoxicity was observed as examined by the WST-1 assay (Roche Diagnostics).

Expression levels of IL-4, IL-6, IL-13, IFN-γ, TGF-β, VEGF, TLR4, TLR7, CCL2, CCL8, CXCL1, CXCL10, and CXCL11 in macrophage culture supernatants from the TLR inhibition studies described above

As portrayed in Fig. 1, 5 mM Omniscan induced significantly increased NF-κB–dependent SEAP production in HEK293 cells expressing either TLR4 or TLR7 compared with control cells incubated with endotoxin-free PBS. Omniscan did not cause statistically significant stimulation of reporter gene expression in the HEK293 cells expressing any of the other TLRs or NLRs. TLR7 expression cells showed a 5-fold increase in SEAP levels compared with the other NLRs in response to Omniscan. These changes in SEAP expression were observed for all replicates for TLR4- and TLR7-expressing cells. Gadodiamide, the Gd chelate in Omniscan, also induced increased NF-κB–dependent SEAP production in HEK293 cells to nearly identical to those induced in response to Omniscan. In contrast to the Gd chelate Omniscan, the macrocyclic Gd

Real-time PCR

Real-time PCR values reflect the mean and SD of three separate experiments each performed in triplicate with each of the three samples of normal human macrophages. The statistical significance of the real-time PCR data was assessed by a Student two-tailed t test. A p value < 0.05 was considered statistically significant.
chelate ProHance did not induce increased NF-κB–dependent SEAP production in HEK293 cells expressing any of the TLRs or NLRs (Fig. 1). Caldiamide and the nonchelated Gd compound Gd-EDTA did not produce significant changes in any TLR or NOD-expressing cell line (data not shown).

**Dose–response analysis of GdBCA signaling through TLR4 and TLR7 in HEK293 cells**

Following identification of TLR4 and TLR7 as participating in Omniscan-induced activation of NF-κB–dependent SEAP production in HEK293 cells, dose–response analysis of the effects of seven Gd chelate compounds and two nonchelated Gd compounds was performed. HEK293 cells expressing either TLR4 or TLR7 were exposed in DMEM complete medium to 0.5, 1, and 5 mM concentrations of the linear GdBCA Gd-DTPA, MultiHance, OptiMark, Omniscan, or gadodiamide; or of the macrocyclic GdBCA Dotarem or ProHance; or to 0.27, 2.7, or 27 μM Gd compounds Gd-EDTA or Gd-citrate; or to 0.025, 0.05, or 0.25 mM non-Gd chelate molecule caldiamide. LPS (1 mg/ml) and gardiquimod (10 mg/ml) were used as positive controls for TLR4 and TLR7 activation, respectively. The concentrations of Gd compounds employed in these studies were similar to those employed in previously published in vitro studies (8, 20, 22–24). Following 24 h exposure to the GdBCA, the levels of NF-κB–dependent SEAP production were measured. The results showed that some of the Gd compounds induced increased SEAP production in a dose-dependent manner. Fig. 2A displays the results for the highest concentration of each agent tested, whereas Fig. 2B shows the three concentration dose–response for Dotarem, MultiHance, Omniscan, and gadodiamide. The linear GdBCA Omniscan and gadodiamide induced a highly significant (p < 0.001) 20-fold increase in NF-κB–dependent SEAP production in TLR4- expressing HEK293 cells and a 16-fold increase in TLR7-expressing HEK293 cells, levels similar to those induced by the LPS-positive control. The linear GdBCA MultiHance and the macrocyclic Dotarem induced much weaker although significant (p < 0.05) 3- to 4-fold increases in SEAP production in TLR4- and TLR7-expressing cells, comparable to the 4-fold increase in SEAP production induced by the nonchelate Gd-EDTA and Gd-citrate. Fig. 2B shows that the response to Dotarem, MultiHance, Omniscan, and gadodiamide was dose-dependent. No response was observed at any concentration examined for the linear GdBCA Gd-DTPA or OptiMark or for the macrocyclic ProHance or of the non-Gd chelate molecule caldiamide (Fig. 2B and data not shown).

**Stimulation of TLR-dependent cytokine, chemokine, and growth factor expression in normal human differentiated macrophages**

To determine whether TLR signaling was required for Omniscan stimulation of macrophage cytokine, chemokine, and growth factor production, normal human macrophages cultured in RPMI 1640 complete medium and differentiated in the presence of M-CSF and IL-10 were preincubated with 50 μM chloroquine or with 300 nM IRAK1/4 inhibitor for 1 h followed by addition of either PBS or PBS containing 1 mM either Omniscan, gadodiamide, or ProHance; or 0.05 mM caldiamide; or 2.7 μM Gd-EDTA for 24 h. As positive controls for TLR-dependent and TLR-independent macrophage stimulation, 100 ng/ml LPS or 10 μg/ml TNF-α, respectively, were used. No significant effect on cell numbers or cytotoxicity was observed as examined by the WST-1 assay. Analysis of expression levels of profibrotic/proinflammatory cytokines by real-time PCR demonstrated that Omniscan and gadodiamide caused markedly upregulated expression of multiple chemokines (Fig. 3) and cytokines and growth factors (Fig. 4). Incubation of macrophages with LPS also induced increases in chemokine, cytokine, and growth factor expression, which were abolished as expected by both inhibitors. The inhibitors, however, failed.

![FIGURE 2](http://www.jimmunol.org/) Comparison of NF-κB–dependent activation by various GdBCA in HEK293 cells expressing TLR4 or TLR7. Measurement of NF-κB–dependent SEAP levels in HEK293 cells expressing a single TLR or NLR at 20 h following exposure in DMEM complete medium to GdBCA. Cells were exposed to 0.5, 1, and 5 mM concentrations of the linear GdBCA Gd-DTPA, MultiHance, OptiMark, Omniscan, or gadodiamide; or of the macrocyclic GdBCA Dotarem or ProHance; or to 0.27, 2.7, or 27 μM Gd compounds Gd-EDTA or Gd-citrate; or to 0.025, 0.05, or 0.25 mM non-Gd chelate molecule caldiamide. LPS (1 mg/ml) and gardiquimod (10 mg/ml) were used as positive controls for TLR4 and TLR7 activation, respectively. Values represent the mean (±SD) absorbance at 650 nm of three replicates of three separate experiments on HEK293 cells in culture. Statistical significance was calculated by comparing each Gd compound to the saline control. Values for other samples are expressed relative to the saline control. (A) SEAP levels secreted in response to exposure to the highest concentration of each agent tested. (B) Three concentration dose–response of SEAP levels secreted following exposure to Dotarem, MultiHance, Omniscan, or gadodiamide. *p < 0.05, **p < 0.01, ***p < 0.001.
to modify the effects induced by TNF-α (data not shown). These results indicated that the stimulation of cytokines, growth factors, and chemokines induced by the GdBCA was mediated by TLRs.

CXCL10 displayed the greatest increase in expression of the chemokines examined, with maximal 20-fold induction by gadodiamide compared with the saline control (Fig. 3). CCL8 expression was induced 16-fold, CXCL11 expression increased 13-fold, CXCL12 expression increased 12-fold, CXCL9 increased 8-fold, and CCL2 showed a 6-fold increase in expression. Gadodiamide induced the strongest levels in expression. A similar pattern was observed for multiple profibrotic/proinflammatory cytokines and growth factors. IL-13 displayed the greatest increase in expression, with a maximal 20-fold induction by gadodiamide at 24 h (Fig. 4). Marked increases in the expression of the growth factors TGF-β (6-fold) and VEGF (5-fold) and the profibrotic cytokines IL-4 (2-fold) and IL-6 (6-fold) were also observed in response to gadodiamide. ProHance had the smallest effect on cytokine and growth factor gene expression. Of interest was the observation that the GdBCA tested induced only minor expression or did not change the levels of IFN-γ, with ProHance inducing only a 2-fold increase, whereas gadodiamide induced a 1.4-fold increase and Omniscan did not change IFN-γ expression levels. Caldiamide did not affect expression levels of any of the genes tested (Figs. 3, 4). Preincubation of macrophages with either chloroquine or IRAK1/4 inhibitor abrogated gadodiamide- and Omniscan-mediated increases in gene expression (Figs. 3, 4).

These results demonstrated that Omniscan and gadodiamide elicited stimulation of potent cytokine, chemokine, and growth factor gene expression in macrophages that is dependent on TLR.

**Inhibition of TLR4 and TLR7 utilizing RNA interference**

To confirm that the observed effects of chloroquine and IRAK 1/4 inhibitors were due to inhibition of the TLR pathway and were not the result of possible effects on other inflammatory pathways, TLR4 and TLR7 expression was directly targeted in normal human differentiated macrophages employing RNA interference. Normal differentiated human macrophages were transfected with control siRNA or with siRNA specific for TLR4 or TLR7 alone or in combination. RNA from these experiments was used in validation of gene expression by real-time RT-PCR of TLR4 and TLR7 (Fig. 5), chemokines (Fig. 6), and proinflammatory/profibrotic cytokines and growth factors (Fig. 7). In control macrophages, the TLR4-specific siRNA induced an 84% decrease in TLR4 mRNA levels without affecting TLR7 expression levels, whereas the TLR7-specific siRNA induced an 88% decrease in TLR7 expression without affecting the RNA levels of TLR4 (data not shown). Exposure of macrophages to 1 mM Omniscan or 1 mM gadodiamide induced TLR4 (Fig. 5A) and TLR7 (Fig. 5B) expression, and this increased expression was reduced to below the levels measured in saline control cells by the specific siRNA. The control siRNA had no appreciable effect on TLR4 or TLR7 expression compared with the saline control. Exposure of cells to either the TLR4-specific siRNA or to the TLR7-specific siRNA prior to treatment with 1 mM Omniscan or 1 mM gadodiamide resulted in abrogation of the GdBCA-induced overexpression of chemokines (Fig. 6) and cytokines and growth factors (Fig. 7), reducing their expression to levels equivalent to or lower than those measured in the saline controls.

**Stimulation of TLR-dependent cytokine, chemokine, and growth factor production in normal human differentiated macrophages**

To confirm that the increased expression observed at the transcript level was reflected at the protein level, SearchLight proteome multiplex arrays were used to quantitate the amounts of relevant profibrotic and proinflammatory cytokines, chemokines, and growth factors.
factors produced by normal human differentiated macrophages following GdBCA exposure alone or following preincubation with chloroquine or IRAK 1/4 inhibitor. The results showed that 24 h exposure to some of the Gd-containing compounds resulted in increased production and secretion of numerous chemokines, cytokines, and growth factors with their significant accumulation in the culture media as shown in Fig. 8. The results, expressed in picograms per milliliter, were normalized for the value of β-actin transcripts obtained in the real-time PCR experiments to correct for possible variance in the number of adherent cells analyzed. All of the cytokines/growth factors that exhibited upregulated mRNA expression following exposure to Omniscan, gadodiamide, ProHance, and Gd-EDTA also demonstrated an increase in the total amount of the corresponding secreted cytokine/growth factor, with the greatest increases seen for cells exposed to Omniscan and gadodiamide. Exposure of macrophages to caldiamide did not result in a detectable increase in the production of any of the cytokines/growth factors analyzed (Fig. 8). As observed for RNA expression levels, preincubation of macrophages with either chloroquine (data not shown) or with IRAK1/4 inhibitor abrogated the Omniscan- and gadodiamide-mediated increased levels of cytokines, chemokines, and growth factors to near baseline levels (Fig. 8).

**Discussion**

Although exposure of patients with renal insufficiency to GdBCA is a primary factor in NSF pathogenesis, the molecular pathways...
stimulated following exposure to GdBCA have not been identified. The data presented in this study demonstrating that Omniscan signals via TLR4 and TLR7 provide a possible mechanism for the initiation of GdBCA-induced inflammation and fibrosis. Omniscan and gadodiamide, the linear Gd chelate present in Omniscan, induce potent stimulation of NF-κB mediated expression of an SEAP reporter protein in HEK293 cells expressing either TLR4 or TLR7 but not in cells expressing one of the other TLRs or the NOD receptors (Figs. 1, 2). The stimulatory effects were comparable to the levels of stimulation induced by the TLR4 ligand LPS or the TLR7 ligand gardiquimod, whereas the chelate molecule caldiamide used in the Omniscan formulation did not stimulate SEAP expression (Fig. 2). One linear GdBCA, MultiHance, showed a much weaker induction of SEAP expression (Fig. 3), as did one of the macrocyclic

**FIGURE 6.** Effect of RNA interference of TLR expression on GdBCA-induced upregulation of chemokine expression in normal differentiated human macrophages. Differentiated normal human macrophages were transfected with 100 nM siGENOME SMARTpool siRNA specific for TLR4, TLR7, or TLR4 plus TLR7 with DharmaFECT 4 (3 μl/well) followed by 24 h incubation with 1 mM Omniscan (Omni), gadodiamide (Gado), or ProHance (ProHan). Expression levels of various chemokines were assessed by real-time PCR. Values represent the mean (±SD) expression levels of three replicates of three separate experiments with macrophages differentiated from monocytes obtained from three different normal individuals. An siGENOME RISC-free control siRNA was used as a negative control. Ct values for chemokines were normalized with β-actin. The saline control levels were arbitrarily set at 100% expression at each time point. Statistical significance of changes in chemokine expression was calculated by comparing each Gd compound to the saline control. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 7.** Effect of RNA interference of TLR expression on GdBCA-induced upregulation of cytokine/growth factor expression in normal differentiated human macrophages. Differentiated normal human macrophages were transfected with 100 nM siGENOME SMARTpool siRNA specific for TLR4, TLR7, or TLR4 plus TLR7 with DharmaFECT 4 (3 μl/well) followed by 24 h incubation with 1 mM Omniscan (Omni), gadodiamide (Gado), or ProHance (ProHan). Expression levels of various cytokines and growth factors were assessed by real-time PCR. Values represent the mean (±SD) expression levels of three replicates of three separate experiments with macrophages differentiated from monocytes obtained from three different normal individuals. LPS (100 ng/ml) and TNF-α (10 ng/ml) were used as positive controls for TLR4 and TNF-α activation, respectively. Ct values for cytokines and growth factors were normalized with β-actin. The saline control levels were arbitrarily set at 100% expression at each time point. Values for other samples are expressed relative to the saline control. Statistical significance of changes in cytokine and growth factor expression was calculated by comparing each Gd compound to the saline control. **p < 0.01, ***p < 0.001.
GdBCA, ProHance, in HEK293 cells. Two nonchelated Gd compounds, Gd-EDTA and Gd-Citrate, stimulated SEAP expression in TLR4- or TLR7-expressing HEK293 cells at a level intermediate between the high levels induced by Omniscan or gadodiamide and the low levels induced by MultiHance or ProHance.

In normal human differentiated macrophages, both Omniscan and gadodiamide strongly induced multiple profibrotic and proinflammatory cytokines, chemokines, and growth factors as measured by real-time PCR of total RNA (Figs. 3, 4) and ELISA of culture supernatants (Fig. 8), whereas ProHance and Gd-EDTA induced a less pronounced increase in expression of these genes. Preincubation of differentiated macrophages with chloroquine or with the IRAK1/4 inhibitor for 1 h abrogated the Omniscan- or gadodiamide-induced expression and production of these proinflammatory/profibrotic molecules (Figs. 3, 4, 8), indicating the crucial role of TLR4 and TLR7 in these effects. These observations were confirmed in experiments targeting TLR4 and TLR7 by RNA interference (Figs. 6, 7), thus conclusively demonstrating that the increased expression levels observed are TLR-dependent. Increased expression induced by TNF-α, which acts downstream of the TLRs, was unaffected by these inhibitors.

Exposure of patients with renal insufficiency to GdBCA is a primary factor in the pathogenesis of NSF (1–4), with the majority of NSF cases attributed to exposure to Omniscan (16, 17). Although the exact mechanisms responsible for the development of NSF following GdBCA exposure in patients with renal insufficiency are not known, there are two hypotheses that have been proposed, as illustrated in Fig. 9. In the transmetallation hypothesis, Gd-containing compounds escape into the extravascular space and transmetallation is induced by endogenous ions allowing free Gd³⁺ to escape. The transmetallation process is dependent on the thermodynamic stability of the GdBCA, and therefore it is more pronounced for the less stable linear chelates (44). The released Gd³⁺ can then escape from the chelate and interact with tissue macrophages, resulting in the production and secretion of proinflammatory/profibrotic cytokines, chemokines, and growth factors by these cells. These secreted macrophage products act on resident tissue fibroblasts, inducing their differentiation into α-smooth muscle actin-expressing myofibroblasts with the consequent increase in production and secretion of a variety of molecules involved in the fibrotic process including the interstitial collagens. The exact mechanisms involved in the escape of Gd-containing compounds into the extravascular space and subsequent transmetallation are not entirely known; however, it is likely that factors such as the higher GdBCA concentrations and their increased retention in the circulation owing to renal insufficiency, associated with alterations in endothelial permeability and tissue edema resulting from inflammatory or thrombotic events, may all contribute to this process (44–46).

In disagreement with the transmetallation hypothesis, however, multiple experimental studies have demonstrated that both linear and macrocyclic GdBCA induce potent metabolic changes in cells, including increased hyaluronan and collagen production by normal human fibroblasts (8, 18–26), increased expression of proinflammatory and profibrotic cytokines, chemokines, and growth factors by normal human PBMCs (27), and increased differentiation of normal human PBMCs into fibrocytes (29). These experimental results have suggested an alternative hypothesis that posits that intact chelated GdBCA are responsible for GdBCA-mediated stimulation (47). In the chelate hypothesis (Fig. 9), the metabolic and molecular events described above are initiated by intact chelated GdBCA rather than by free Gd³⁺. The cellular pathways exploited by either chelated GdBCA or transmetallated free Gd³⁺ to initiate these...
FIGURE 9. Diagramatic representation of two possible pathways that may participate in the induction of NSF by GdBCA. This schematic diagram represents two possible mechanisms by which GdBCA result in activation of target cells. In the transmetallation model, free Gd$^{3+}$ is displaced from the chelate complex by endogenous ions, such as Zn$^{2+}$, allowing it to stimulate proinflammatory and profibrotic responses in macrophages and fibroblasts. In the chelate model, these effects are mediated by chelated GdBCA, which have been retained in the body due to severely reduced clearance rates in patients with renal insufficiency. In both models, the cellular recognition of either free Zn$^{2+}$ (transmetallation model) or of the intact chelated GdBCA (chelate model) is mediated through TLR4 and TLR7 signaling.

Cellular changes is largely unknown. The data presented in this study suggest an important role of TLR signaling in NSF pathogenesis in particular and for the development of fibrosis in general. First, the data suggest that the ability to trigger TLR signaling is more potent following exposure to Omniscan and gadodiamide. The most obvious explanation is that these molecules possess a unique, specific molecular shape or pattern that renders them capable of signaling via TLR4 and TLR7. Caldiamide, the chelate molecule contained in Omniscan, failed to activate NF-$\kappa$B, and Gd-EDTA activated only weakly NF-$\kappa$B, suggesting that the greatest effect on TLR signaling is mediated by an intact Omniscan Gd chelate molecule. Although the molecular pattern responsible for TLR activation is not known, it is possible that the formation of coordination bonds with Gd$^{3+}$ apparently alters the nonstimulatory molecular pattern of caldiamide sufficiently to render the chelate complex capable of activating TLR signaling.

Omniscan and gadodiamide signaling via both TLR4 and TLR7 allows these compounds to exert their effects at the cell surface and in the endosome. Endosomal TLR signaling is particularly relevant for macrophages owing to the stimulatory effect of TLR signaling on phagocytosis by macrophages (48). Initial engagement of TLR4 by Omniscan at the macrophage cell surface could induce and increase the rates of Omniscan phagocytosis. Once phagocytosed, Omniscan would be able to amplify its TLR signaling capacity by engaging TLR7 within the endosome. Several reports of Gd deposits found in affected tissues from NSF patients described the presence of macrophages in close proximity (9–12), suggesting that the highly acidic environment of the endosome could facilitate deposition of Gd salts, such as Gd phosphate and Gd carbonate (49). These precipitated Gd salts could then maintain a constant state of TLR activation in both macrophages and fibroblasts, producing a chronic proinflammatory/profibrotic phenotype responsible for disease persistence and progression. Gd salts could act as a secondary trigger for the innate immune system, preventing the normal resolution of the TLR response following GdBCA clearance, inducing the release of profibrotic cytokines, chemokines, and growth factors.

Fibroblasts express TLR4 at the cell surface, and it is possible that some of the direct effects of Gd compounds on fibroblasts such as inducing their differentiation into $\alpha$-smooth muscle actin-expressing myofibroblasts and the increased production and secretion of molecules involved in the fibrotic process such as collagen and hyaluronan could be due to engagement of fibroblast TLRs. We are currently investigating whether Gd compounds are capable of engaging normal fibroblast TLR signaling. It is also possible that GdBCA-induced hyaluronan could also signal via TLR4 expressed on macrophages and fibroblasts, contributing to a chronic proinflammatory/profibrotic environment (39).

Taken together, the results described in this study demonstrate that Omniscan and gadodiamide signal through TLR4 and TLR7, resulting in the increased expression of genes encoding several well-characterized profibrotic and proinflammatory cytokines, chemokines, and growth factors in normal human differentiated macrophages. The role of TLR4 and TLR7 in this stimulation was confirmed by the abrogation of these effects by specific TLR4 and TLR7 inhibitors as well as by RNA interference studies. The present study provides strong evidence and plausible pathophysiological mechanisms for initiation and maintenance of a proinflammatory/profibrotic phenotype by Omniscan and gadodiamide in the development of NSF. The marked ability of Omniscan and gadodiamide to signal through TLR4 and TLR7 compared with nonchelated Gd compounds and the inability of the chelate backbone, caldiamide, to induce this response suggest that the intact Gd chelate complex is capable of initiating innate immunity activation and that the specificity of the stimulation may be dependent on an intrinsic molecular pattern present in this chelate molecule. Further study and characterization of the cellular effects of these compounds and of the mechanisms of these effects may provide valuable information regarding the early events in the pathogenesis of NSF and other fibrosing diseases such as SSc.

Disclosures
The authors have no financial conflicts of interest.

References
