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**INDUCTION OF A TYPE I INTERFERON SIGNATURE IN NORMAL HUMAN MONOCYTES BY GADOLINIUM-BASED CONTRAST AGENTS: COMPARISON OF LINEAR AND MACROCYCLIC AGENTS**

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## **Abstract**

The gadolinium-based contrast agent (GdBCA) Omniscan activates human macrophages through TLR4 and TLR7 signaling. To explore the mechanisms responsible we compared the ability of linear and macrocyclic GdBCA to induce a type I interferon signature and a proinflammatory/profibrotic phenotype in normal human monocytes *in vitro*. Expression of genes associated with type I interferon signaling and inflammation, and production of their corresponding proteins were determined. Both linear and macrocyclic GdBCA stimulated expression of multiple type I interferon regulated genes and the expression of numerous chemokines, cytokines and growth factors in normal human peripheral blood monocytes. There was no correlation between the magnitude of the measured response and the Gd chelate used. To explore the mechanisms responsible for GdBCA induction of fibrosis in Nephrogenic Systemic Fibrosis (NSF) *in vitro*, normal human dermal fibroblasts were incubated with GdBCA-treated monocyte culture supernatants and the effects on profibrotic gene expression were examined. Supernatants from monocytes exposed to all GdBCA stimulated types I and III collagen, fibronectin and  $\alpha$ -SMA expression in normal dermal fibroblasts. The results indicate that the monocyte activation induced by GdBCA may be the initial step in the development of GdBCA associated fibrosis in NSF.

## Introduction

Nephrogenic Systemic Fibrosis (NSF) is a systemic fibrosing disorder occurring in patients with chronic kidney disease (CKD) characterized by progressive fibrosis of skin, periarticular tissues, striated muscles and various internal organs (1-4). Histopathological studies of affected NSF skin demonstrate severe dermal and subdermal fibrosis, prominent mucin deposition, accumulation of activated fibroblasts and macrophages, and strikingly increased *in situ* expression of transforming growth factor beta (TGF- $\beta$ )(3). Although the exact mechanisms responsible for NSF development are not known the majority of reported cases occurred following exposure to high doses of Gd based contrast agents (GdBCA) in the context of renal insufficiency (5-8). Gd accumulation in CKD patient tissues is thought to be the primary event in NSF pathogenesis. Indeed, numerous studies have described Gd deposition in affected tissues from NSF patients (9-11).

Two models that differ in the nature of the bioactive form of Gd associated with NSF occurrence in CKD individuals have been proposed (12-16). The transmetallation model posits that GdBCA accumulate in the extravascular space and tissues where endogenous free cations such as  $Zn^{2+}$ ,  $Ca^{2+}$ ,  $Cu^{2+}$ , or  $Fe^{2+}$  displace  $Gd^{3+}$  from the chelate complex (12-15). The  $Gd^{3+}$  released from the chelate complex can induce activation of various profibrotic molecular pathways in one or more of the cell types present in fibrotic NSF lesions, such as macrophages, fibroblasts and fibrocytes. According to this model, less thermodynamically stable linear GdBCA are more prone to undergo transmetallation thereby initiating the fibrotic process leading to NSF. The second model, the chelate model, proposes that intact Gd chelate complexes, rather than transmetallated  $Gd^{3+}$ , are the

bioactive agent triggering the NSF fibrotic process (16). This model suggests that certain intrinsic property(s) of the intact Gd chelate complex is(are) responsible for the disproportionately greater association of linear GdBCA with NSF development compared to macrocyclic GdBCA based on exposure rates to these classes of agents.

Activated macrophage infiltration into affected NSF skin and visceral tissues (3) and the induction of a proinflammatory/profibrotic phenotype in normal human peripheral monocytes and macrophages by the linear contrast agents Omniscan and Gd-DTPA have been previously described (17,18). Recently, it has been reported that Omniscan stimulation of production of proinflammatory/profibrotic cytokines, chemokines and growth factors is mediated by TLR4 and TLR7 in normal differentiated human macrophages (19). Numerous other studies have demonstrated that GdBCA can induce profibrotic responses in normal and NSF fibroblasts as well as in skin organ cultures (20-23). However, the induction of a Type I interferon signature, and a detailed comparison of proinflammatory and profibrotic effects of various linear and macrocyclic GdBCA have not been performed. Thus, the purpose of the present study was to compare the ability of four different linear GdBCA and two different macrocyclic GdBCA to induce a Type I interferon signature and the expression of a proinflammatory/profibrotic phenotype in normal human monocytes *in vitro*. Furthermore, the effects of supernatants from these cells on cultured normal human dermal fibroblasts were examined to explore the mechanisms that might be responsible for the differential association of various GdBCA agents with NSF development.

## **Experimental**

**Gd compounds.** Dotarem<sup>®</sup> (Guerbet LLC, Bloomington, IL) , MultiHance<sup>®</sup> (Bracco Diagnostics, Milan, IT), ProHance<sup>®</sup> (Bracco Diagnostics, Milan, IT), OptiMark<sup>®</sup> (Mallinckrodt Inc/Covidien, Hazelwood, IN), were supplied as sterile, aqueous solutions each containing 500 mM of the Gd chelate. Omniscan<sup>®</sup> and gadodiamide (provided by GE Healthcare, Chalfont St. Giles, UK) were supplied as sterile, aqueous solutions each containing 287 mg/ml (500 mM) gadodiamide. The Omniscan<sup>®</sup> 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  $\mu$ M) was prepared by mixing 1 mL of 250  $\mu$ M gadolinium chloride and 1 mL of 500  $\mu$ M sodium citrate solutions at pH 7.4 (24). Gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA; Sigma-Aldrich. St. Louis, MO), the Gd chelate used in Magnevist<sup>®</sup>, was dissolved in sterile PBS solution at 0.5M concentration. The reagents employed for all the studies were tested and verified by the manufacturer to be free from endotoxin contamination. The absence of endotoxin contamination was further confirmed in our laboratories utilizing the Etoxate Assay (Sigma-Aldrich) according to the manufacturer's instructions.

**Monocyte Isolation.** Leukoreduction filters from three different normal donors were obtained from the Thomas Jefferson University Hospital Blood Bank following Institutional Review Board approval. Human peripheral blood mononuclear cells (PBMC) were isolated from the filters by Ficoll-Hypaque gradient centrifugation (Amersham

Pharmacia Biotech, Piscataway, NJ) and enriched for monocytes by adherence to plastic culture dishes for 2 h as described (17,25).

**Treatment of monocytes with Gd compounds.** Equal numbers of monocytes were plated in 60 mm plastic culture dishes in RPMI 1640 media supplemented with 25 mM HEPES, L-glutamine and 10% FBS. Lipopolysaccharide (LPS; 1  $\mu$ g/ml) was the positive control and sterile saline was the negative control. Cells were exposed for 12 h or 24 h to 5 mM of each of the six GdBCA or to 250  $\mu$ M caldiamide which corresponds to the amount present in the Omniscan® preparation. Total RNA was isolated from the cells exposed to GdBCA for 12 h as previously described (17,18) and supernatants from each sample were collected and stored at -20°C until used.

**Enzyme-linked immunosorbent assay.** SearchLight ELISA proteome array analyses (Pierce Biotechnology, Woburn, MA) were performed as described previously (17,18) to quantitate the levels of IL-4, IL-6, IL-13, IFN- $\gamma$ , TGF- $\beta$ , and VEGF in supernatants from monocytes exposed to the Gd compounds for 24 h. Briefly, samples were diluted 1:2, 1:50, and 1:1,000 and then incubated for 1 h on the array plates which had been prespotted with capture antibodies specific for each protein. Plates were decanted and washed 3 times with PBS before addition of a mixture of biotinylated detection antibodies to each well. Following incubation with detection antibodies for 30 min, plates were washed 3 times and incubated for 30 min with streptavidin–horseradish peroxidase. Plates were again washed and SuperSignal Femto chemiluminescent substrate was added. The plates were immediately imaged using the SearchLight imaging system and data were analyzed using ArrayVision software.

**Real-time polymerase chain reaction (PCR).** Monocyte and fibroblast transcript levels were quantified using SYBR Green real time PCR, as previously described (17,18). Primers were designed using Primer Express software (Applied Biosystems, Foster City, CA) and validated for specificity. The primers employed are displayed in Supplemental Table 1. The differences in the number of mRNA copies in each PCR were corrected for human  $\beta$ -actin endogenous control transcript levels; levels in control experiments were set at 100 and all other values expressed as multiples of control values.

**Treatment of cultured fibroblasts with conditioned media from peripheral blood monocytes exposed to Gd compounds.** Two normal human dermal fibroblast cell lines (passages 4-10) from the Scleroderma Center Tissue Bank, Thomas Jefferson University, were cultured in Dulbecco's modified Eagle's (MEM) medium supplemented with 10% fetal bovine serum, antibiotics, 50 mM HEPES, and glutamine until confluent.

For experiments, the cells were cultured in complete medium in 6-well plastic plates until confluence. Following preincubation for 24 h with 40  $\mu$ g/ml ascorbic acid (Sigma-Aldrich) to optimize collagen production, fibroblasts were incubated for 24 h with conditioned media from Gd-exposed peripheral blood monocytes at a 1:2 dilution. Fibroblasts were then harvested with trypsin-EDTA, washed with PBS, and then processed for RNA extraction using the RNeasy kit (Qiagen, Valencia, CA) as described previously (17,18).

**Statistical analysis.** Real-time PCR values reflect the mean and standard deviation 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 Student's two-tailed t test. *P* values less than 0.05 were considered statistically significant.



## Results

**Effects of Gd compounds on cytokine and growth factor gene expression levels in normal human monocytes.** Expression levels of profibrotic/proinflammatory cytokines in normal human monocytes in response to 12 h exposure to Gd-containing agents were analyzed by real time PCR. IL-6 and IL-13 were markedly increased in response to all Gd compounds whereas VEGF was increased by all Gd compounds except MultiHance and OptiMARK (**Figure 1**). IL-4 was increased by all Gd compounds except Gd-DTPA, MultiHance and OptiMARK whereas TGF $\beta$  was increased only in response to Omniscan, gadodiamide, Gd-EDTA and Gd-Citrate. IFN- $\gamma$ , however, was increased only in response to Gd-DTPA, Dotarem and ProHance. No correlation between the level of induction of expression of various genes encoding the proinflammatory/profibrotic cytokines and growth factors and the structure of the GdBCA chelate molecule or the non-chelated Gd compound was observed. Exposure of monocytes to caldiamide had no effect on the expression level of any gene measured.

**Effects of Gd compounds on chemokine gene expression levels by normal human monocytes.** Expression levels of chemokine genes in normal human monocytes in response to 12 h exposure to Gd-containing agents were analyzed by real time PCR. CXCL9 and CXCL12 were markedly increased in response to all Gd compounds whereas CCL2 was increased by all Gd compounds except Gd-DTPA and CCL8 was increased by all Gd compounds except OptiMARK (**Figure 2**). CXCL11 was increased by all Gd compounds except MultiHance and Dotarem whereas CXCL10 was increased only in response to Gd-DTPA, Omniscan, gadodiamide, Gd-EDTA and Gd-Citrate. As with

expression levels of cytokines and growth factors, no correlation between the level of response attained and the structure of the chelate molecule of the GdBCA was observed.

**Effects of Gd compounds on normal human monocyte production of proinflammatory/profibrotic cytokines and chemokines.** To assess whether the effects observed at the gene expression level were also reflected at the protein level, the amounts of cytokines and growth factors present in culture supernatants isolated from normal human monocytes incubated for 24 h with the Gd compounds were assayed by SearchLight proteome multiplex arrays (**Figure 3**). The levels of IL4, IL13, and TGF $\beta$  produced were significantly elevated by all of the GdBCA tested, regardless of the chelate class (**Figure 3**), and all agents except for OptiMARK induced increased VEGF levels whereas only Omniscan, gadodiamide and ProHance significantly induced increased production of IL-6. Omniscan and gadodiamide treated cells produced the highest levels of IL-4, IL-6, and IL-13, whereas, Omniscan, gadodiamide and MultiHance produced the highest levels of VEGF.

**Conditioned media from Gd-stimulated normal human monocytes induce a profibrotic phenotype in normal human dermal fibroblasts.** Fibroblasts cultured with supernatants from monocytes that had been exposed to linear and macrocyclic GdBCA or to nonchelated Gd compounds displayed increased expression of type I and type III human collagen (COL1A and COL3A) respectively. All monocyte supernatants caused collagen gene expression stimulation with similar efficacy (**Figure 4**). Similarly,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) expression, a marker of myofibroblast activation, and fibronectin (FN1) expression were upregulated by all of the GdBCA, although supernatants from cells

exposed to the linear chelate GdBCA showed a slightly greater level of upregulation of  $\alpha$ -SMA expression compared to those induced by macrocyclic GdBCA. In contrast, supernatants from monocytes exposed to caldiamide alone did not affect expression of any of the genes examined in the cultured fibroblasts.

**Effects of Gd compounds on expression levels of type I inteferons and of genes involved in induction of NF $\kappa$ B by normal human monocytes.** Expression levels of type I interferons (IFN- $\alpha$  and IFN- $\beta$ ) and of the NF $\kappa$ B activator genes, Interferon Regulatory Factor 1 (IRF1), IRF4, IRF9 and X-linked Inhibitor of Apoptosis (XIAP) were analyzed in normal human monocytes following 12 h exposure to Gd-containing agents. All linear chelates and non-chelated Gd compounds induced striking increases in the expression of all 6 genes (**Figure 5**). In contrast, Dotarem induced increased expression of IFN- $\alpha$ , IFN- $\beta$ , IRF4 and IRF9 but not of IRF1 or XIAP and ProHance increased expression of only IFN- $\alpha$ , IFN- $\beta$  and IRF9.

**Effects of Gd compounds on gene expression levels of interferon-responsive genes by normal human monocytes.** Analysis of expression levels of interferon-responsive genes in normal human monocytes in response to 12 h exposure to Gd-containing agents were analyzed next. Expression of CCL20, Interferon Inducible 44-like (IFI44L), Interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), IL8, IL18 and Immunoresponsive gene 1 (IRG1) were all markedly increased in response to all GdBCA (**Figure 6**). No correlation was observed between the level of response attained and the structure of the chelate molecule of the GdBCA with all linear and macrocyclic GdBCA.

We also found that all non-chelated Gd compounds induced expression of all interferon-responsive genes.

## Discussion

Exposure to GdBCA is a crucial event in NSF pathogenesis but the mechanisms that trigger this severe fibrotic disease are poorly understood. Multiple, non-mutually exclusive hypotheses to explain the roles of a variety of molecular pathways and possible cofactors in NSF pathogenesis have been proposed (reviewed in 12-16, 26, 27). One area of debate concerns the form of the active Gd molecule. The transmetallation hypothesis proposes that free  $Gd^{3+}$  is the active form (12-15) whereas the chelate hypothesis posits the intact Gd chelate molecule instead (16). The present study was designed to compare the relative abilities of linear and macrocyclic GdBCA to induce a proinflammatory/profibrotic phenotype in normal human monocytes. A second goal of the studies described was to examine the ability of the linear and macrocyclic GdBCA to affect expression levels of type I IFNs and type I IFN responsive genes as well as genes involved in NF $\kappa$ B expression and NF $\kappa$ B-mediated transcriptional activation. These studies were carried out in order to provide a mechanistic link between the previously described GdBCA signaling through TLR4 and TLR7 (19) and the induction of the profibrotic/proinflammatory phenotype responsible for NSF development.

The results demonstrate that both classes of GdBCA as well as non-chelated Gd compounds induce in normal human monocytes the expression and secretion of numerous cytokines, chemokines and growth factors generally recognized as important mediators of the initiation and/or progression of the fibrotic process. Further, culture supernatants isolated from monocytes exposed to either linear or macrocyclic agents or to non-chelated

Gd compounds were shown to stimulate type I and type III collagens and  $\alpha$ -SMA and FN1 expression in normal human dermal fibroblasts. While we recognize that HEPES buffer may negatively affect B-lymphocyte DNA replication, several studies have found that this effect is observed only in the absence of CO<sub>2</sub> (28) and that in the presence of CO<sub>2</sub> HEPES offers greater buffering capacity and ensures better cell survival and stimulation of cellular proliferation (29). Indeed, in a previous study we observed comparable levels of stimulation of gene expression by GdBCA in human macrophages cultured in the presence of HEPES to the results described here in human lymphocytes (19). The changes in expression of numerous proinflammatory/profibrotic chemokines, cytokines and growth factors and of type I interferon responsive genes were consistent in three separate experiments utilizing monocytes derived from three different normal individuals. However, the magnitude of the observed effects was variable among the cells from the three individuals, possibly owing to genetic heterogeneity and variability in the sensitivity of monocytes from different donors to the activating stimuli. These observations are consistent with epidemiological studies showing that NSF development occurs infrequently even among the large population of renal insufficiency patients exposed to GdBCA.

There was no correlation between the induction of proinflammatory/profibrotic gene expression in normal human monocytes with the dynamic stability of the GdBCA as would be expected if the active agent was free Gd<sup>3+</sup>. While some free Gd<sup>3+</sup> release cannot be entirely ruled out for ProHance, the thermodynamic stability of Dotarem at the acidic pH levels found in macrophage lysosomal compartments is ~338 h (27). Therefore, if free Gd<sup>3+</sup> is required for the induction of profibrotic molecular pathways as proposed in the

transmetallation hypothesis, then the macrocyclic agents should have had minimal or no detectable effects on cytokine and growth factor expression. Thus, the greater ability of certain GdBCA to induce NSF cannot be explained simply to their enhanced susceptibility to undergo transmetallation. It seems more likely that a unique characteristic of certain linear GdBCA or of gadodiamide in particular, such as its shape, molecular pattern, or molecular surface reactivity is responsible for the disproportionately high frequency of NSF cases associated with their use.

It has recently been reported that Omniscan and gadodiamide induce TLR4 and TLR7-dependent expression of profibrotic/proinflammatory cytokines and growth factors in normal human differentiated macrophages (19). The results described here demonstrate that all Gd compounds examined were capable of inducing expression of genes associated with TLR signaling, including XIAP, which activates NFκB expression in response to TGFβ signaling (30), and of IRF1, IRF4 and IRF9 which are components of downstream intracellular signaling complexes mediating NFκB target gene regulation and induction of type I IFN expression (31-34). Interestingly, exposure to all Gd compounds tested resulted in a striking induction of the expression of type I interferon responsive genes CCL20, IFI44L, IFIT, IL8, IL18 and IRG1. These observations are consistent with the notion that GdBCA molecular pattern recognition by the TLR as well as a direct effect of free Gd<sup>3+</sup> participate in NSF pathogenesis.

These results, therefore, suggest the establishment of a signaling pathway in which GdBCA as well as non-chelated Gd compounds signal through TLR4 and TLR7, inducing

expression of XIAP and IRF genes that mediate NF $\kappa$ B activation. NF $\kappa$ B activation and signaling, in turn, induce the expression of type I interferon responsive genes that mediate the establishment of a proinflammatory/profibrotic phenotype characterized by the expression and production of cytokines, chemokines and growth factors capable of triggering the activation of quiescent fibroblasts into myofibroblasts. This putative pathway is illustrated in **Figure 7**. These results are of relevance to other fibrotic disorders such as Systemic Sclerosis (SSc) in which activation of a type I interferon pathway has been reported (35-41).

In conclusion, the results described here demonstrate that GdBCA, regardless of chelate class and thermodynamic stability, induce in normal human monocytes remarkably potent expression of numerous genes encoding well-characterized profibrotic/proinflammatory chemokines, cytokines and growth factors and of type I interferons and interferon-responsive genes resulting in their increased production and release. Furthermore, conditioned medium from monocytes exposed to the different Gd-containing compounds caused a potent stimulation of expression of the profibrotic markers types I and III collagen, fibronectin and  $\alpha$ -SMA in normal human dermal fibroblasts. The observations that both linear and macrocyclic classes of Gd chelates as well as the non-chelated Gd-containing compounds induce these potent cellular phenotypic and gene expression changes in normal human macrophages indicate that GdBCA thermodynamic stability is not likely to be the primary factor influencing the relative activity of these compounds in the induction of NSF.



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**Disclosures**

The authors have no financial conflicts of interest.

## Figure Legends

**Figure 1. Effect of non-ionic and ionic linear and macrocyclic GdBCA on the expression levels of various genes encoding profibrotic/proinflammatory cytokines and growth factors in normal human monocytes.** Values represent the mean (+/- SD) expression levels of three replicates of three separate experiments with monocytes obtained from three different normal individuals exposed to the Gd compounds for 12 h. C(t) values for cytokines were normalized with  $\beta$ -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. Expression levels of (A) IL4, (B) IL6, (C) IL13, (D) IFN $\gamma$ , (E) TGF $\beta$ , and (F) VEGF transcripts are displayed. Statistical significance of changes in cytokine and growth factor expression as determined by Student's T test comparing each Gd compound to the saline control appears above the corresponding bar. \*: p<0.05; \*\*: p < 0.01; \*\*\*: p<0.001. Caldiamide did not induce a significant effect for any cytokine or growth factor.

**Figure 2. Effect of non-ionic and ionic linear and macrocyclic GdBCA on the expression levels of various chemokine genes in normal human monocytes.** Values represent the mean (+/- SD) expression levels of three replicates of three separate experiments with monocytes obtained from three different normal individuals exposed to the Gd compounds for 12 h. C(t) values for cytokines were normalized with  $\beta$ -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. Expression levels of (A) CCL2, (B) CCL8, (C) CXCL9, (D) CXCL10, (E) CXCL11, and (F) CXCL12 transcripts are displayed. Statistical significance of changes in chemokine expression as determined by Student's T test comparing each Gd compound to the saline control appears above the corresponding bar. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ . Caldiamide did not induce a significant effect for any cytokine or growth factor.

**Figure 3. Quantitative measurement of various cytokines and growth factors present in the culture media of Gd-compound-exposed normal human monocytes employing multiplex proteome array analyses.** Cells were incubated with either saline, 5 mM Gd-DTPA, MultiHance, Omniscan, gadodiamide, Dotarem or ProHance, or 2.5 mM caldiamide for 24 h and the culture media was assayed. Values are expressed in pg/ml and represent the mean (+/-) value of values obtained from duplicate results of pooled samples of 3 individuals at 3 dilutions: 1:2, 1:50 and 1:1000. Statistical significance of changes in cytokine and growth factor expression as determined by Student's T test comparing each Gd compound to the saline control appears above the corresponding bar. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ . Caldiamide did not induce a significant effect for any cytokine or growth factor.

**Figure 4. Effects of supernatants from monocytes exposed to Gd compounds on expression of types I and III collagen, fibronectin and  $\alpha$ -SMA in normal dermal human fibroblasts.** Fibroblasts were incubated for 24 h in media containing a 1:2 dilution

of culture supernatants isolated from monocytes exposed to Gd compounds for 12 h. Expression levels of (A) COL1A1, (B) COL3A1, (C) FN1 and (D)  $\alpha$ -SMA transcripts determined by real time PCR. The results shown are representative of three separate experiments, each performed in triplicate. The saline control levels were arbitrarily set at 100%. Values for the samples are expressed as the mean (+/- SD) percent increase over the saline control value. Statistical significance of changes in cytokine and growth factor expression as determined by Student's T test comparing each Gd compound to the saline control appears above the corresponding bar. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ . Supernatants from monocytes exposed to caldiamide did not induce a significant effect on fibroblast gene expression.

**Figure 5. Effects of Gd compounds on expression of XIAP and IRF family genes in normal human monocytes.** Values represent the mean (+/- SD) expression levels of three replicates of three separate experiments with monocytes obtained from three different normal individuals. C(t) values for cytokines were normalized with  $\beta$ -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. Expression levels of (A) IRF1, (B) IRF4, (C) IRF9, and (D) XIAP transcripts are displayed. Statistical significance of changes in chemokine expression as determined by Student's T test comparing each Gd compound to the saline control appears above the corresponding bar. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ . Caldiamide did not induce a significant effect.

**Figure 6. Effects of Gd compounds on expression of type I interferon responsive genes in normal human monocytes.** Values represent the mean (+/- SD) expression levels of three replicates of three separate experiments with monocytes obtained from three different normal individuals. C(t) values for cytokines were normalized with  $\beta$ -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. Expression levels of (A) CCL20, (B) IFI44L, (C) IFIT1, (D) IL8, (E) IL18, and (F) IRG1 transcripts are displayed. Statistical significance of changes in chemokine expression as determined by Student's T test comparing each Gd compound to the saline control appears above the corresponding bar. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ . Caldiamide did not induce a significant effect.

**Figure 7. Pathways mediating GdBCA induction of proinflammatory/profibrotic phenotype in normal human macrophages.** Cellular recognition of either free  $Gd^{3+}$  (transmetallation model) or of the intact chelated GdBCA (chelate model) is mediated through TLR4 and TLR7 signaling. The signaling events result in upregulation of XIAP and IRF genes and activation of NF $\kappa$ B which cooperate to upregulate type I interferons, that in turn activate type I interferon signature genes (ISGs) resulting in production of proinflammatory and profibrotic chemokines, cytokines and growth factors. These factors are secreted by activated macrophages and can then act to promote the transdifferentiation of quiescent fibroblasts to activated myofibroblasts, characterized by initiation of  $\alpha$ -SMA expression and in dysregulated and sustained extracellular matrix production leading to tissue fibrosis. Figure modified from (19).

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