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Fibroblast Response to Lanthanoid Metal Ion Stimulation: Potential Contribution to Fibrotic Tissue Injury

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Abstract

The purpose of this study was to compare each of the 14 naturally occurring lanthanoid metal ions for ability to stimulate pro-fibrotic responses in human dermal fibroblasts. When fibroblasts were exposed to individual lanthanoids over the concentration range of 1–100 μ M, increased proliferation was observed with each of the agents as compared with control cells that were already proliferating rapidly in a growth factor-enriched culture medium. Dose-response differences were observed among the individual metal ions. Matrix metalloproteinase-1 (MMP-1) and tissue inhibitor of metalloproteinase-1 levels were also increased in response to lanthanoid exposure but type I procollagen production was not. A dose-response relationship between induction of proliferation and increased MMP-1 was observed. Non-lanthanoid transition metal ions (aluminum, copper, cobalt, iron, magnesium, manganese, nickel, and zinc) were examined in the same assays; there was little stimulation with any of these metals. When epidermal keratinocytes were examined in place of dermal fibroblasts, there was no growth stimulation with any of the lanthanoids. Several of the lanthanoid metals inhibited keratinocyte proliferation at higher concentrations (50–100 μ M).

Keywords

Fibroblast; Lanthanoid metal ions; Nephrogenic systemic fibrosis (NSF); Matrix metalloproteinase-1 (MMP-1); Tissue inhibitor of metalloproteinase-1 (TIMP-1); Type I procollagen

Introduction

The lanthanoid elements constitute a group of cationic metals with atomic numbers between 57 and 71. These closely related elements have an ionic radius similar to that of Ca^{2+} but with a higher overall charge density [1, 2]. Lanthanoid ions bind to Ca^{2+} -binding sites on proteins, and in some cases, the binding affinity is greater than that of Ca^{2+} itself [3]. The extracellular calcium-sensing receptor (CaSR) is a target protein in epithelial cells [4–7]. Calcineurin is another important regulatory molecule influenced by lanthanoid metal ions [8]. Lanthanoids also impact the functioning of Ca^{2+} channels, including receptor-activated channels, voltage-gated channels, and those responsive to mechanical stress [9–11].

Past studies have demonstrated reduced epithelial cell proliferation and increased differentiation in the presence of lanthanoid metals [12, 13], and this is thought to reflect

interaction with CaSR. Epithelial growth reduction is not universal, however, as increased proliferation and decreased differentiation have also been reported [14, 15]. Lanthanoid-induced growth arrest has also been seen with melanoma cells [16], but the underlying molecular mechanism is not known. In cells with inflammatory function, including macrophages, stimulation through Toll-like receptors is observed, leading to altered production of pro-inflammatory and pro-fibrotic cytokines [17]. Interestingly, in whole animals, both cytokine stimulation and inhibition have been reported [18, 19].

Certain lanthanoids including lanthanum (La^{3+}), cerium (Ce^{3+}), lutetium (Lu^{3+}), and gadolinium (Gd^{3+}) have been shown to stimulate proliferation in fibroblasts [12, 20-26]. Our own studies have shown that Gd^{3+} -induced fibroblast growth is accompanied by up-regulation of matrix metalloproteinase-1 (MMP-1) and tissue inhibitor of metalloproteinase-1 (TIMP-1) [12, 24]. The level of TIMP-1 is sufficiently high in the Gd^{3+} -exposed cells that MMP-1 activity is not detectable, and there is, concomitantly, increased collagen deposition into the cell layer without a change in procollagen synthesis [27].

Lanthanoid exposure is associated with tissue fibrotic changes in various settings [28-39], but the mechanistic events that lead to fibrosis are not completely understood. The capacity of several lanthanoids to induce fibrotic changes under one condition or another, coupled with their ability to induce responses in fibroblasts that are, potentially, pro-fibrotic, provided the impetus for the current study. The question addressed here is whether the lanthanoid metal ions that have been associated with fibrotic tissue changes are unique in their ability to induce pro-fibrotic responses in dermal fibroblasts, or whether all of the lanthanoids share similar properties. To address this question, we have obtained chloride salts of each lanthanoid metal (with the exception of promethium which is not available as a naturally occurring element) and assessed responses in human dermal fibroblasts to each element over a wide range of concentrations. Human epidermal keratinocytes were examined in parallel as control.

Materials and Methods

Lanthanoid Metal Ions

Fourteen different lanthanoid metal ions were used in the present study. These included La^{3+} , Ce^{3+} , praseodymium (Pr^{3+}), neodymium (Nd^{3+}), samarium (Sm^{3+}), europium (Eu^{3+}), Gd^{3+} , terbium (Tb^{3+}), dysprosium (Dy^{3+}), holmium (Ho^{3+}), erbium (Er^{3+}), thulium (Tm^{3+}), ytterbium (Yb^{3+}), and Lu^{3+} . Each one was obtained as the chloride salt (Sigma Chemical Co.; St. Louis, MO) and made up to 500 mM in distilled water. In addition to the lanthanoids, we also assessed a number of non-lanthanoid divalent and trivalent metal ions. Included were aluminum (Al^{3+}), copper (Cu^{2+}), cobalt (Co^{2+}), iron (Fe^{2+} and Fe^{3+}), magnesium (Mg^{2+}), manganese (Mn^{2+}), nickel (Ni^{2+}), and zinc (Zn^{2+}). These non-lanthanoids were also obtained from Sigma Chemical Company as chloride salts.

Other Reagents

A rabbit polyclonal antibody to MMP-1 (AB19040) for use in Western blotting and a mouse monoclonal antibody to E-cadherin (MAB 3197) for use in Western blotting and confocal immunofluorescence microscopy were obtained from Millipore/Chemicon (Temecula, CA). Human synovial fibroblast MMP-1 was obtained from Calbiochem (San Diego, CA).

Human Dermal Fibroblasts and Epidermal Keratinocytes

Human foreskin tissue was obtained from circumcisions performed at the University of Michigan Hospitals. De-identified tissue samples were obtained under an exemption from

institutional review board oversight. This tissue was used to establish cultures of dermal fibroblasts as described previously [40]. 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. The HaCat line of immortalized human epidermal keratinocytes [41] was used as a source of epithelial cells. The keratinocytes were grown in keratinocyte growth medium (KGM; Lonza). KGM consists of MCDB-153 as base but is supplemented with a mixture of growth factors including hydrocortisone, epithelial growth factor, insulin, and bovine pituitary extract. The Ca^{2+} concentration of KGM is 0.15 mM. Maintenance of both fibroblasts and keratinocytes was at 37°C in an atmosphere of 95% air and 5% CO_2 . Cells were routinely used at passage 2–3.

Fibroblast Proliferation

Fibroblasts were added to wells of a 24-well dish at 4×10^4 cells per well in DMEM-FBS and allowed to attach. The wells were then washed two times with 1 mL of KGM supplemented with extracellular Ca^{2+} to a final concentration of 1.5 mM. Our past studies have demonstrated that, when KGM is supplemented with extracellular Ca^{2+} to this concentration, it provides a potent fibroblast growth stimulus [40]. After washing, duplicate wells were counted to provide accurate “zero-time” values. One milliliter of Ca^{2+} -supplemented KGM was added to each remaining well. Each of the lanthanoid metal ions (and non-lanthanoids) was added at 1–100 μM final concentration. 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 MMP-1, TIMP-1, and type I procollagen as described below. Cells were harvested with trypsin–ethylenediaminetetraacetic acid (EDTA) and counted using an electronic particle counter.

Cytotoxicity and Apoptosis Assays

To assess viability after lanthanoid exposure, fibroblasts were incubated with the lanthanoid for 1 day as above and then harvested and counted. Immediately following this, cytotoxicity and apoptosis were assessed by staining with Annexin V-FITC and propidium iodide, and by analyzing stained cells via flow cytometry [42]. Briefly, cells were harvested, washed twice with ice-cold phosphate-buffered saline, and then resuspended in $1 \times$ binding buffer (BD Pharmingen, San Diego, CA) at a concentration of 1×10^6 cells/mL. Two hundred microliters of the cell suspension was transferred to wells of a 96-well V-bottom plate. Ten microliters of Annexin V-FITC (BD Pharmingen, San Diego, CA) and 5 μL of propidium iodide (Invitrogen Molecular Probes, Carlsbad, CA) were added to each well and incubated for 15 min in the dark. Samples were then analyzed by flow cytometry (LSR II, BD Biosciences, San Diego, CA). Data acquisition and analysis were done using BD FACSDiva software.

MMP-1 Analysis

Western blotting with a rabbit polyclonal anti-MMP-1 antibody was used to assess MMP-1 levels [12, 24]. Briefly, samples consisting of conditioned medium were separated in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing and reducing conditions and transferred to nitrocellulose membranes. After blocking with 5% nonfat milk solution in Tris-buffered saline with 0.1% Tween (TTBS) at 4°C overnight, membranes were incubated for 1 h at room temperature with anti-MMP-1 antibody, diluted 1:1,000 in 5% nonfat milk/TTBS. Thereafter, the membranes were washed with TTBS and the bound antibody detected using the Phototope-HRP Western blot detection kit (Cell Signaling Technologies, Inc., Beverly, MA). Images were scanned, digitized, and quantified. Prior to loading the gels, protein levels in each sample were determined using the bicinchoninic acid (BCA) protein determination kit (Pierce Biotechnology, Rockford, IL), and equal amounts of protein were loaded onto each lane. In some experiments, the Ponceau

S reversible staining solution was used to verify equal protein transfer from the gels to the Western blot membranes. In some experiments, a standard curve was generated using purified human fibroblast-derived MMP-1 and the values were compared with the standard curve.

MMP-2 Analysis

The same culture fluids were assessed for matrix metalloproteinase-2 (MMP-2; gelatinase A) by gelatin zymography [43]. Briefly, SDS-PAGE gels were prepared with the incorporation of gelatin (1 mg/mL) at the time of casting. After electrophoresis under non-reducing conditions to separate proteins and overnight incubation to allow for substrate digestion, zones of hydrolysis were identified as “holes” in the stained gels and then quantified. Values for latent and active MMP-2 were obtained following scanning and digitization. MMP-2 tends to be expressed at a basal level under most conditions in fibroblasts. Therefore, MMP-2 can be used as a control for secreted proteins in much the same way that actin or β -tubulin is utilized as a control for intracellular proteins.

TIMP-1 Analysis

Culture fluids were assayed for TIMP-1 by enzyme-linked immunosorbent assay (ELISA) using a commercially available assay kit (R&D Systems, Minneapolis, MN) [12].

Type 1 Procollagen Analysis

Culture fluids were assayed for type I procollagen by ELISA using a commercially available assay kit (Takara, Madison, WS) [12]. 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.

Keratinocyte Proliferation

Proliferation studies with keratinocytes were carried out as with fibroblasts, except that the growth medium consisted of KGM with a final Ca^{2+} concentration of 0.15 mM for maintenance of cells in the undifferentiated condition (unless otherwise stated) or 1.5 mM to promote differentiation. Keratinocytes were routinely harvested and counted after 2 days of incubation.

E-cadherin Analysis: Preparation of Cell Lysates and Immunoblot

Keratinocytes were plated at 3×10^5 cells per well in six-well tissue culture dishes and then allowed to attach. Following attachment, the cells were incubated for 1 day in KGM with the desired amount of Ca^{2+} and Gd^{3+} as described in the Results section. The next day, cultures were washed and then lysed in $1 \times$ cell lysis buffer consisting of 20 mM Tris-HCl (pH 7.4), 2 mM sodium vanadate, 1.0 mM sodium fluoride, 100 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 25 $\mu\text{g/mL}$ each of aprotinin, leupeptin, and pepstatin, and 2 mM EDTA and EGTA. Lysis was performed by adding 200 μL of lysis buffer to each well and incubating the plate on ice for 5 min. After incubation, cells were scraped and samples were sonicated. Then, the extracts were cleared by microcentrifugation at $14,000 \times g$ for 15 min. Supernatants were collected and then protein concentrations estimated using the BCA protein determination kit. Western blotting for E-cadherin was carried out essentially as described above with MMP-1.

E-cadherin Analysis: Confocal Immunofluorescence Microscopy

Keratinocytes were added to Lab Tek II chamber slides and then allowed to attach. Following attachment, the cells were incubated in KGM containing 0.15 and 1.5 mM Ca^{2+} with or without 50 μM Gd^{3+} . The next day, cultures were washed and then fixed with 4%

formaldehyde for 20 min. After fixation, cells were washed 2× with wash buffer [0.05% Tween-20 in Dulbecco's phosphate-buffered saline (DPBS)], followed by permeabilization with 0.1% Triton X-100 for 5 min. Cells were again washed and then exposed to a blocking solution consisting of 1% bovine serum albumin in DPBS for 30 min. Next, cells were treated with a monoclonal antibody to E-cadherin in blocking solution for 1 h. After three subsequent washing steps with DPBS (5 min each), cells were treated with Alexa Fluor 488-conjugated rabbit anti-mouse IgG antibody (Invitrogen) in blocking solution and then incubated for 30 min. Following three additional washing steps, the cells were treated with Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody in the same solution to amplify the fluorescence signal. Finally, the cells were rinsed once with wash buffer and then mounted with Prolong Gold anti-fade medium containing DAPI (Invitrogen). Cells were examined with a Zeiss LSM 510 confocal microscope using a ×63 (C-Apochr) NA=1.2 water immersion objective lens. Laser excitation wavelengths included 364, 488, and 543 nm scanned in sequence by the line method.

Statistical Analysis

Data were analyzed using one-way analysis of variance followed by the Bonferroni post-test for selected pairs (GraphPad Prism version 4.00 for Windows, GraphPad Software). Data were considered significant at $p < 0.05$. Asterisks have been added to the appropriate bars in the figures and individual values in the tables to denote values that are significantly higher than the respective control value.

Results

Lanthanoid Metal Ion-Induced Fibroblast Proliferation

Each of the 14 naturally occurring lanthanoid metal ions was examined for ability to stimulate dermal fibroblast growth over a wide range of concentrations. The data from these studies are shown in Fig. 1. Each of the metal ions stimulated proliferation, but as can be seen from the individual panels, there were significant differences in concentration-dependence and maximal stimulation. The most active agents consisted of two separate group of elements in the middle of the series (Sm^{3+} , Eu^{3+} , and Gd^{3+} and Ho^{3+} , Er^{3+} , and Tm^{3+}) separated by lanthanoids (Tb^{3+} and Dy^{3+}) with lower activity. Lanthanoids on either end of the series were also of lower activity. With the most active members, optimal proliferation was seen at a concentration as low as 5 μM . With these agents, the net increase in cell number was greater than twofold over control level.

At high concentrations (50 and 100 μM), certain of the lanthanoids produced a fall-off in cell number. In all cases, the lanthanoids that induced a growth response at 5 and 10 μM produced the greatest fall-off at 50 and 100 μM . With 100 μM Eu^{3+} , Gd^{3+} , Ho^{3+} , Er^{3+} , and Tm^{3+} , the number of cells remaining at day 3 was significantly lower than the number present at the start of treatment. Annexin V-propidium iodide was used to verify cell death and to determine apoptotic changes in lanthanide-exposed fibroblasts. For this, fibroblasts were exposed to Gd^{3+} (50 or 100 mM) and then compared with control cells. As seen in Table 1, the fall-off in cell number was associated with cell death, but there was little increase in apoptotic cells as compared with control.

MMP-1, TIMP-1, and Type I Procollagen

At the time of harvest, i.e., after 72 h of incubation, serum-free culture medium was collected from fibroblasts treated with a subset of lanthanoids. Among these were two elements shown to be highly active in the proliferation assay (Sm^{3+} , Eu^{3+}) as well as elements with intermediary activity (Ce^{3+} , Pr^{3+} , and Nd^{3+}) and a lanthanoid (La^{3+}) with low activity in the proliferation assay. The culture fluids were examined for MMP-1 by Western

blotting. The enzyme was strongly up-regulated by each of the agents (Fig. 2). The values shown in the figure are based on densitometry. When the densitometry values obtained with the culture fluids were compared directly with densitometry values obtained from a standard curve generated using commercially obtained human fibroblast MMP-1, culture fluid values ranged from less than 1 ng mL^{-1} in the controls to greater than 10 ng mL^{-1} with optimal stimulation (not shown). A concentration-dependent relationship between MMP-1 up-regulation and induction of proliferation can be seen (compare Figs. 1 and 2). In subsequent experiments (not shown), culture fluids from fibroblasts exposed to each of the other lanthanoids were also assessed for MMP-1. Each of these lanthanoids also induced MMP-1 at concentrations that were stimulatory for proliferation. As a control for MMP-1, we also assessed lanthanoid effects on levels of MMP-2. Previous studies have demonstrated that MMP-2 is not modulated by factors that up-regulate MMP-1 [43]. Other than a fall-off in MMP-2 levels at cytotoxic concentrations, there was no effect on this enzyme (not shown).

Lanthanoid concentrations that supported proliferation and MMP-1 production were next assessed for TIMP-1 and type I procollagen levels. Results are summarized in Table 2, where it can be seen that TIMP-1 was increased with each of the agents, while lanthanoid exposure had little net effect on type I procollagen levels. These results with the complete series of lanthanoid metal ions confirm findings obtained earlier with Gd^{3+} alone [12, 24].

Human Epidermal Keratinocyte Proliferation in Response to Lanthanoid Metal Ions

Each of the 14 lanthanoid metal ions was examined for effects on epidermal keratinocyte proliferation (Fig. 3). To summarize, there was no growth enhancement with any of the metals. With certain of the metals, a reduction in growth as compared with control was seen at 50 and/or 100 μM . However, cell numbers never fell below zero-time values. The data depicted in Fig. 3 were obtained using KGM supplemented with 0.15 mM Ca^{2+} as the growth medium. Similar results were obtained when the Ca^{2+} concentration was reduced to 0.035 mM (lowest level that maintained cell viability) or when the level of extracellular Ca^{2+} was increased to 1.5 mM (i.e., a Ca^{2+} concentration that promoted differentiation).

Experiments were carried out to determine if keratinocyte growth reduction was accompanied by a measurable effect on differentiation. For this, keratinocytes were treated with 50 μM Gd^{3+} and examined for E-cadherin expression by Western blotting and by confocal immunofluorescence microscopy. As seen in Fig. 4, there was no measurable change in E-cadherin expression when the cells were exposed to 50 μM Gd^{3+} under low- Ca^{2+} (0.15 mM) conditions. As expected, in the presence of 1.4 mM Ca^{2+} , differentiation (increased total E-cadherin and increased surface expression) was induced. This was not measurably enhanced by Gd^{3+} .

Effects of Non-lanthanoid Metal Ions on Fibroblast and Keratinocyte Proliferation

As part of these studies, human dermal fibroblasts were examined for proliferation in response to a series of non-lanthanoid metal ions. Among the metal ions tested were Al^{3+} , Co^{2+} , Cu^{2+} , Fe^{2+} , Fe^{3+} , Mg^{2+} , Mn^{2+} , Ni^{2+} , and Zn^{2+} . These metals have been associated with fibrotic changes in the lungs following inhalation exposure [44, 45]. Results from these studies are summarized in Table 3. With most of these non-lanthanoid metal ions, there was no induction of proliferation. Where proliferation was seen—i.e., with aluminum and iron—the response was observed at the higher concentrations (50 and 100 μM), was modest (maximal increase of approximately 35%), and was not statistically different from control.

The same non-lanthanoid metal ions examined with fibroblasts were also tested for effects on keratinocyte proliferation. None of these metal ions significantly stimulated proliferation over the concentration range of 1–100 μM (Table 3). Inhibition was observed with certain of

the ions at higher concentrations. The greatest suppression was observed with 100 μM Co^{2+} (35% reduction) and 100 μM Zn^{2+} (approximately 18% below control value).

Discussion

From the standpoint of basic chemistry, the lanthanoids are all similar to one another [1, 2]. Not surprisingly, their effects on biological systems are also quite similar. For example, in a study of algae response to different lanthanoids, Tai et al. [46] reported that each of 13 different lanthanoids produced growth inhibition over a similar concentration range. A mix of lanthanoids produced the same growth suppression as individual elements at the same overall molar concentration. The conclusion from this study was that the “simple” biological system was unable to distinguish among the closely related moieties. Several studies have described qualitatively similar but quantitatively different responses to individual lanthanoids. For example, variation has been reported in regard to protein stabilization in aqueous solution [47], reduction in lacunae formation by osteoclasts [48], and growth modulation in certain mammalian cell lines [14-16]. Other than to note differences among the elements for modulating one cell function or another, there is little that points to a particular chemical property as being critical. One of the problems is that few previous studies have examined all of the lanthanoids together (much less, over a wide dose range). Perhaps, if all 14 of the naturally occurring lanthanoids were studied together (as in the present work), chemical and biological relationships could be seen more clearly. Even under the best of conditions, however, interpretation of biological results might be difficult as no one chemical property is likely to explain all of the changes in the complex biological phenomena observed. This is, in fact, suggested from the current study where there was only a partial overlap between stimulation of fibroblasts and growth inhibition in keratinocytes.

With regard to effects on human dermal fibroblasts, we and others have shown that Gd^{3+} is able to induce proliferation [12, 23-26]. Associated with growth induction is up-regulation of MMP-1 and TIMP-1 without a change in type I procollagen. The end result is the increased deposition of collagen into the cell layer/extracellular matrix [27]. Based on the data presented here, Gd^{3+} does not appear to be unique in its ability to stimulate responses in human dermal fibroblasts. Quite the contrary, the current manuscript demonstrates that, while there are quantitative differences among the various lanthanoids, all 14 naturally occurring lanthanoid ions have the capacity to stimulate events in human fibroblasts that may contribute to fibrotic tissue injury. That only certain of the lanthanoid species have been associated with fibrotic disease to date [28-39] may reflect quantitative differences among the various lanthanoids in their effects on fibroblast function but may also reflect differences in exposure. It should be noted that not all fibroblasts appear to be equally sensitive to lanthanoid stimulation. A previous study demonstrated that cerium was able to stimulate proliferation in cardiac fibroblasts under conditions in which lung fibroblasts did not respond [49]. Furthermore, our own studies showed that, among fibroblast isolates from adult human subjects, there was a spectrum of responsiveness to Omniscan, one of the Gd^{3+} -containing magnetic resonance imaging contrast agents [50]. Responsiveness was associated with a functional platelet-derived growth factor (PDGF) receptor and signaling through the mitogen-activated protein kinase pathway [24, 51]. Failure of some cells to mount an active response may reflect a loss of signaling capacity through this pathway. In support of this, past studies have demonstrated a loss of PDGF responsiveness in fibroblasts as a consequence of aging [52]. Thus, failure of certain fibroblasts to respond to lanthanoid-induced growth stimulation may reflect the physiological state of the cells in question rather than a unique response to the lanthanoids.

In contrast to results with the lanthanoid metal ions, none of the non-lanthanoid elements examined here induced similar changes in fibroblast function. Based on that, it is unlikely

that the non-lanthanoid metal ions induce fibrosis through a direct stimulation of fibroblast proliferation. Exposure to dusts and particles of various metals is associated with fibrotic changes in the lung [44, 45]. The generally held view is that phagocytosis of inhaled particles by tissue inflammatory cells leads to generation of oxidants and pro-inflammatory cytokines, which, in turn, promote inflammation and tissue damage [44, 45, 53]. As part of the repair process (normal or fibrotic), resident fibrogenic cells, as well as circulating precursors (fibrocytes), are activated by inflammatory mediators to differentiate into myofibroblasts with enhanced collagen-producing capability [54-56]. Our data do not rule out lanthanoids acting in a similar fashion [57, 58]. Our data simply suggest that this may not be the only pathway to fibrotic changes. With the lanthanoids, direct stimulation of collagen-producing cells may be as important as activating inflammatory pathways. The lung, of course, is unique in its exposure to dusts and small particles. Fibrosis associated with lanthanoid exposure has been seen in other tissues where such exposure would not be expected [28-39, 59], as well as in the lung.

In summary, this study examined the 14 naturally occurring lanthanoids (as well as several non-lanthanoids) for effects on human skin cells under ex vivo conditions. The effects observed with the lanthanoid metal ions on fibroblast function were significantly different from effects seen with the non-lanthanoids. Both lanthanoid and non-lanthanoid metal ions are capable of inducing fibrotic changes, though induction of fibrogenic changes may occur through different mechanisms.

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Fig. 1. Fibroblast proliferation in response to lanthanoid metal ions. Human dermal fibroblasts were treated with each of the lanthanoid metal ions at time-zero and counted after 3 days. Values from controls were set to 100, and values obtained with each concentration of lanthanoid were compared with control. Values are means and standard deviations based on three or more trials with each metal ion. *Asterisks* indicate statistically significant increased cell number relative to control at $p < 0.05$ level. Control proliferation index (i.e., the number of cells at day 3 divided by the number of cells at day 0 was 2.3 ± 0.3)

Fig. 2.

MMP-1 levels in culture fluids from lanthanoid-treated fibroblasts. Human dermal fibroblasts were treated with each of the lanthanoid metal ions at time-zero. Culture fluids were harvested on day 3 and assayed for MMP-1 by Western blotting. Bands were scanned and digitized. Values from controls were set at 1.0 and values obtained with each concentration of lanthanoid compared with control. Each lanthanoid was assessed in three or more trials with similar results

Fig. 3.

Keratinocyte proliferation in response to lanthanoid metal ions. Human epidermal keratinocytes were treated with each of the lanthanoid metal ions at time-zero and counted after 2 days. Values from controls were set to 100 and values obtained with each concentration of lanthanoid were compared with control. Values are means and standard deviations based on three or more trials with each metal ion. Control proliferation index (i.e., the number of cells at day 2 divided by the number of cells at day 0 was 3.1 ± 0.2)

Fig. 4. Keratinocyte differentiation in response to lanthanoid metal ions. Human epidermal keratinocytes were incubated with 0.15 or 1.5 mM Ca²⁺ with and without 50 μ M Gd³⁺. Two days later, cells were assessed for E-cadherin expression by Western blotting (*upper panel*) or by confocal fluorescence microscopy (*lower panel*). Western blots: bands were scanned and digitized. Values from control (0.15 mM Ca²⁺) were set at 1.0 and other values compared with control. The experiment was carried out five times with similar results. Confocal fluorescence microscopy: **a** 0.15 mM Ca²⁺, **b** 0.15 mM Ca²⁺+50 μ M Gd³⁺, **c** 1.5 mM Ca²⁺, **d** 1.5 mM Ca²⁺+50 μ M Gd³⁺

Table 1

Gadolinium cytotoxicity

Treatment	Analysis	
	Percent dead (Propidium iodide)	Percent apoptotic (Annexin V)
Control	<5	<5
Gd ³⁺ (μM)		
1	<5	<5
5	<5	<5
10	<5	<5
50	6±1	8±1
100	33±4	9±2

Fibroblasts were exposed to Gd³⁺ and incubated for 1 day. At the end of the incubation period, cells were harvested and counted to obtain a total cell count. Cells were then treated with Annexin V and propidium iodide and then examined by flow cytometry. The total cell count was used in conjunction with the number of Annexin V-positive and propidium iodide-positive cells to obtain a measure of dead cells and a measure of cells undergoing apoptosis but not yet dead

Values are means and standard deviations based on duplicate samples in two independent experiments

Table 2

TIMP-1 and type I procollagen levels in culture fluids from lanthanoid-treated fibroblasts

Lanthanoid	TIMP-1	Type I procollagen
Control	100	100
Lanthanum	120±15	108±5
Cerium	133±23 [*]	108±8
Praseodymium	149±34 [*]	112±15
Neodymium	121±22	97±12
Samarium	121±20	107±2
Europium	130±28 [*]	107±14

Fibroblasts were treated with the indicated lanthanoid at 1–100 μ M and incubated for 3 days in Ca^{2+} - supplemented KGM. The values shown in the table were from concentrations that provided maximal response. Control values were set at 100, and all other values were normalized to the control value. Values are means and standard deviations based on results from a single experiment with triplicate data points. Each lanthanoid was examined two or three times with similar results. TIMP-1 control, 108±32 ng/mL. Type I procollagen control, 1,520±88 ng/mL.

^{*}
 $p < 0.05$ level indicating statistically significant increase relative to control

Table 3
Fibroblast and keratinocyte proliferation in response to selected non-lanthanoid metals

Metal, μM	Al^{3+}	Fe^{2+}	Fe^{3+}	Co^{2+}	Cu^{2+}	Ni^{2+}	Mg^{2+}	Mn^{2+}	Zn^{2+}
Fibroblasts									
Control	100	100	100	100	100	100	100	100	100
1	105	88	105	110	99	109	102	96	104
5	94	104	102	109	83	108	99	99	106
10	101	105	118	105	80	11	99	88	111
50	110	131	122	101	95	94	90	92	97
100	135	133	130	74	105	88	87	99	95
Keratinocytes									
Control	100	100	100	100	100	100	100	100	100
1	103	104	102	95	100	100	93	90	101
5	100	100	105	93	89	110	91	99	104
10	99	100	106	98	87	109	98	98	96
50	108	100	104	69	94	105	99	103	98
100	115	102	104	65	93	96	94	97	82

Human dermal fibroblasts and human epidermal keratinocytes were treated with each of the metal ions at time-zero and counted after 3 days (fibroblasts) or 2 days (keratinocytes). Values from controls were set to 100% and the values obtained with each concentration of each metal compared with control. Values are means based on three separate trials with duplicate or triplicate samples per trial for each metal ion. Standard deviations were within 15% of the means, and no concentration of agent induced a statistically significant increase relative to control