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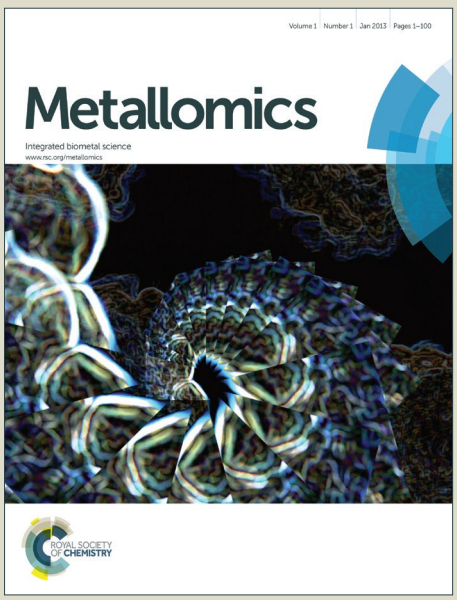

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ARTICLE

The gadolinium-based contrast agent Omniscan® promotes *in vitro* fibroblast survival through *in situ* precipitation

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The current study aimed to explore how gadolinium (Gd)-based contrast agent (GBCA) Omniscan® enhanced cell viability of murine fibroblasts. The results showed that Omniscan® can precipitate in cell culture media and deposit on cell membranes by scanning electron microscopy. Energy-dispersive X-ray analysis and Fourier-transform infrared spectroscopy demonstrated the presence of Gd and phosphates in the agglomerated particles. By filtering the Omniscan®-containing media through a 220-nm filter, it clearly indicated that the increased cell viability should be mainly attributed to the insoluble species of the gadolinium rather than to the chelated gadolinium. Moreover, the effects of other gadolinium-based contrast agents, Magnevist® and Dotarem® were compared with that of Omniscan®. It is noted that the three contrast agents differed in the potency of their ability to induce cell viability, which is possibly ascribed to their different chemical stability of gadolinium chelates as demonstrated by the attenuation in cell growth with the addition of excess ligands of the compounds. The results also showed that Omniscan® can promote cell growth via an increase in the S-phase cell population by flow cytometry analysis evidenced by the elevated levels of cell cycle associated proteins cyclin D, cyclin A and phosphorylated Rb protein. Furthermore, our results revealed that integrin-mediated signaling may play an important role in both Omniscan® and Magnevist®-enhanced focal adhesion formation since the blockade of integrins decreased the level ERK phosphorylation induced by the two GBCAs. Taken together, these data suggested that *in situ* gadolinium phosphate precipitation formation mediated Omniscan®-promoted fibroblast survival, which is similar to that of gadolinium chloride. It demonstrated that the application of GBCAs with more stable thermodynamic stability may cause less dissociation of gadolinium ion and thus resulted in less precipitation, finally may lead to lower occurrence of nephrogenic systemic fibrosis. The obtained results would be also helpful for the development of safe gadolinium-based contrast agents.

Introduction

The causal link between nephrogenic systemic fibrosis (NSF) and the gadolinium(Gd)-based contrast agents (GBCAs) has been established [1-3]. Currently, the prevailing hypothesis is that, in patients of renal dysfunction, increase of the plasma elimination half-life of Gd-based contrast agents may lead to the dissociation of Gd ions by *in vivo* transmetallation [3, 4]. The dissociated Gd^{3+} may be present in the insoluble form and would be phagocytosed by macrophages and subsequently induced cytokines and chemokines release which may recruit fibrocytes into tissues where they would differentiate into fibroblasts [5, 6], thus leading to an overproduction of collagen [7-9]. Furthermore, our previous work and others also indicated the proliferative effects of insoluble Gd-containing particles, formed in cell culture media whether by addition of gadolinium chloride or by exposure directly to insoluble gadolinium phosphate, on the fibroblasts [10-12]. The chelated gadolinium in the form of Omniscan®, Magnevist®, MultiHance®, and ProHance® have also been reported to increase proliferation of human dermal fibroblasts directly [12-14]. Nevertheless, whether intact GBCA themselves or their transformed species in the medium are responsible for the fibroblast proliferation is still unclear. Owing to their strong binding to biologically available anions (phosphate, carbonate, hydroxide, etc), soluble Gd ions tend to form insoluble salts in biological fluids [15, 16]. The regular cell culture medium usually contains 0.91 mM PO_4^{3-}, which is similar to the concentration of phosphate in blood [17], so it is reasonable to speculate that dechelation process may occur during the incubation period when GBCAs were exposed to cells in the cell culture media. We then hypothesize that Gd-based

contrast agent would form insoluble deposits and exert the effects via a mechanism similar to that of gadolinium chloride. As has been shown in our previous studies, gadolinium chloride instantly formed into precipitation in the culture medium, which can promote cell survival and G1 to S phase transition by enhancing focal adhesion formation via integrin-mediated signaling pathway [10, 11, 18]. Therefore, our aim here is to investigate whether *in situ* gadolinium phosphate transformation of GBCAs occurred in the cell culture medium. Meanwhile, the present study will determine whether the formed insoluble deposits are capable of promoting fibroblast survival and the underlying mechanism will also be examined. The mouse embryonic 3T3-L1 fibroblasts are used as a model system, which are frequently used to study the mechanisms of cell proliferation [19, 20]. The results obtained may not only aid to the development and application of Gd-based contrast agents but also for prevention and treatment for NSF.

Materials and methods

Materials

Dimethyl sulfoxide, gadolinium chloride hexahydrate (purity 99.999%), Propidiumiodide, 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ribonuclease, vinculin antibody， and RGD (Arg-Gly-Asp) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO. Calf serum was from Hyclone. Antibodies against glyceraldehyde 3-phosphate dehydrogenase, GAPDH, phosphorylated ERK 1/2 (Thr-202/Tyr-204), horseradish peroxidase labeled rabbit anti-mouse immunoglobulin G (IgG) and phosphorylated retinoblastoma tumor-suppressor protein (ppRb at Ser780) were from Cell Signaling Technology (Beverly, MA, USA). Anti-cyclin A, anti-cyclin D antibody and integrin blocking antibodies against integrins α5 and β1 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG was from Zhongshan Jinqiao Company (Beijing, China). Amersham ECL Plus™ western blotting detection reagents were obtained from GE Healthcare companies (Amersham, UK). Rhodamine-labeled phalloidin and Hoechst 33258 were from Molecular Probes (Eugene, OR, USA). For Gd compounds, Gadodiamide (Omniscan®), Gadopentetate dimeglumine (Magnevist®) and Gadoterate meglumine (Dotarem®) were obtained from GE Healthcare (Amersham, UK), Bayer Schering Pharma (Berlin, Germany) and Guerbet (Paris, France), respectively. Unless otherwise stated, we used the trade names of the three GBCAs throughout the manuscript. $GdCl_3$ solution was prepared by dissolving solid gadolinium chloride hexahydrate in 0.9% NaCl solution and was diluted to 50 mM $GdCl_3$. The RGD stock solution was prepared with 0.1 M acetic acid at a concentration of 20 mg/ml. All other reagents were of analytical grade.

Cell culture

Mouse-derived 3T3-L1 cells were obtained from the Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% new born calf serum (CS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37℃and 5% CO_2 atmosphere. Except for cell synchronization, all other cell treatments were performed in DMEM containing 1% CS to maintain cell activities. While in the process of cell synchronization, 3T3-L1 cells were grown to 90% confluence and then rendered quiescent by incubation in DMEM containing 0.1% CS for 15 h.

MTT assay

Exponentially growing 3T3-L1 cells were seeded in 96-well microplates at a density of 5,000 cells per well and were grown to about 70% confluence. The cells were exposed to $GdCl_3$ or gadolinium-based agents for certain periods as indicated. Then, the medium was aspirated and substituted with the tetrazolium salt MTT solution with a concentration of 0.5 mg/ml. The cells were incubated for an additional 4 h at 37 ℃. Then MTT was removed and dimethyl sulfoxide (200 μl/well) was added to dissolve the formazan crystals for 30 min. The absorbance at 595 nm was measured on a microplate reader (TECAN SUNRISE, Switzerland). The method of integrin blocking experiment has been described previously [10]. Briefly, 3T3-L1 cells were pre-treated with blocking antibody against integrin α5 (1 μg/10^6 cells), integrin β1 (2 μg/10^6 cells), or RGD (0.4 mg/ml) for 1 h. The percentage of cell viability was compared with cells treated at 0 h. The cell viability was calculated by normalizing the absorbance to the corresponding control.

Scanning electron microscopy and energy dispersive X-ray analyses

Cells were seeded on cover slips for 24 h, then exposed to Omniscan® (2 mM) in DMEM containing 1% CS for 24 h. The cells were fixed with 3% glutaraldehyde in phosphate-buffered saline (PBS). After dehydration in a series of graded ethanol, the samples were treated with gold and viewed with a scanning electron microscope (SEM) (JEOL, JSM-5600 LV). An energy dispersive X-ray (EDX) microanalyzer (DX-4X, PHILIPS) attached to SEM was used to determine the elemental content.

Infrared spectroscopy

The infrared spectrum was recorded in the range of 400–4000 cm^{-1} with an FTIR spectrometer (Nexus 470, Thermo Nicolet Corporation). Gd-containing particles were dispersed in spectroscopically pure KBr pellets prepared by gently mixing 1 mg of the sample with 200 mg of KBr.

Flow cytometry analysis

Cell synchronization was performed for 19 h in DMEM containing 0.1% CS. Then the synchronized cells were incubated with gadolinium-based agents at the intervals indicated. At the end of exposure time, the cells were trypsinized by 0.25% trypsin in PBS and washed twice with cold PBS. Then the cells were kept in ice-cold 75% ethanol at -20 ºC overnight. The fixed cells

were resuspended and washed once with cold PBS followed by incubation for 30 min at room temperature in PBS containing 7.5 μM/mL propidium iodide (PI) and 100 μg/mL ribonuclease (RNase A). The cellular DNA contents were assayed with a FACS-Calibur flow cytometer (Becton Dickinson, USA) and the cell cycle distribution was analyzed by using ModFit software program (Beckton Dickenson).

Immunofluorescence microscopy

After 24 h of 3T3-L1 cell culture in six-plate wells, cells were synchronized and then exposed to Omniscan® and Magnevist® for 18 h or were left untreated. Afterwards, cells were fixed in 4% paraformaldehyde. After two washes of PBS, cells were treated with PBS containing 0.2% Triton X-100 (TPBS). The 3T3-L1 cells were incubated with the primary antibody against vinculin at a dilution of 1:200 and followed by incubation with FITC-conjugated rabbit anti-mouse IgG at a dilution of 1:400. While for F-actin and nuclei, PBS containing 2 U/ml rhodamine-labeled phalloidin and 10 μg/ml Hoechst 33258 was added. Images were taken with a confocal laser scanning microscope (CLSM) (TCS SP2; Leica, Germany).

Western blot analysis

The western blot analysis was performed as described in our previous study [18]. Briefly, the cell pellets were collected and fully lysed in cold RIPA buffer [50 mM tris(hydroxymethyl)aminomethane-HCl, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM $NaVO_3$, 1 mM NaF, 1 mM phenylmethylsul-fonyl fluoride, pH 7.4] for 20 min. Then, samples were centrifuged for 20 min and protein content was determined by Bradford protein assay. The protein was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to a poly(vinylidene difluoride) membrane (Millipore Cooperation), and probed with antibodies against the proteins of interest. The bands were visualized using ECL-Plus according to the manufacturer's instructions.

Statistics

Data are presented as the mean ± the standard deviation. Statistical analyses were performed by Student's t test, with $P < 0.05$ indicating statistical significance.

Results

In the cell culture medium, gadolinium-based contrast agent Omniscan® precipitated and deposited on the cell membranes

To determine whether *in situ* of dissociation of the Omniscan® may occur in the cell culture medium, cells were observed by SEM after incubation of with Omniscan® for 24 h. As seen in Fig.1, no particles were seen on the cells in the absence of Omniscan® (Fig.1a). But in the presence of Omniscan®, agglomerated particles were seen on the cell surface (Fig.1b and 1c), similar to that of gadolinium chloride [11]. By EDX analysis (Fig. 1d), the presence of Gd is indicated. Using FT-IR, we further determined the composition of the precipitation in the medium. As seen in Fig 1. e, the wide bands in the region 3000-3700 cm^{-1} were attributed to water vibrations. Importantly, the absorption bands exhibited active vibrations of PO_4^{3-} centered at 1066 cm^{-1} and 541 cm^{-1} as the black arrows indicated. In addition, the spectrum also exhibited apparent absorption bands centered at 1654 cm^{-1}, 1533 cm^{-1} and 1463 cm^{-1} which can be assigned to amide I, II, and III C=O stretching modes, respectively, indicating a possible modification of amino acids residue-containing constituents including serum proteins in the culture media.

In summary, we showed here that Omniscan®, as a Gd chelate characterized by a relatively poor thermodynamic stability, converted into insoluble Gd phosphates in the cell culture medium and deposited on the cell membrane.

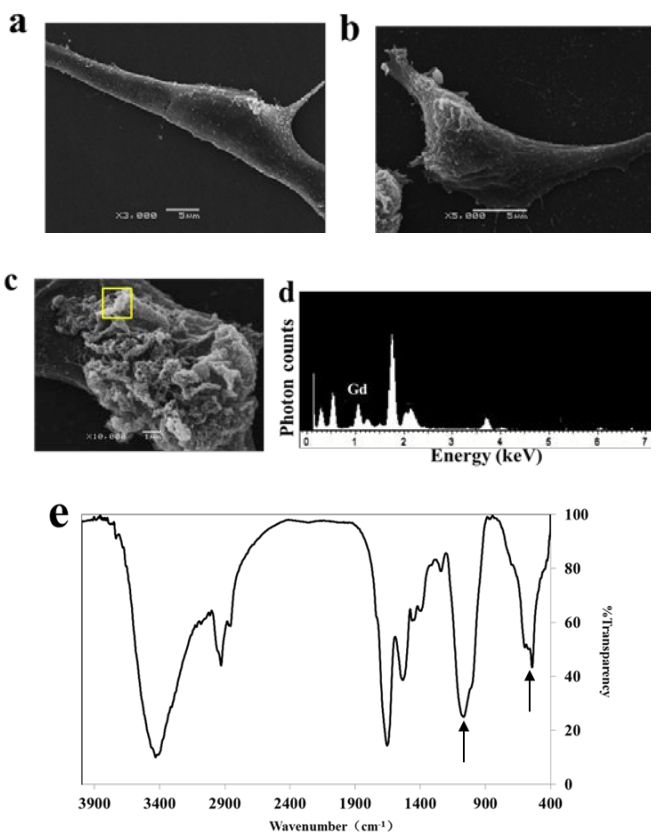


Fig 1. Gadolinium-based contrast agents Omniscan® formed insoluble Gd phosphates and deposited on the cell surface in the cell culture medium. 3T3-L1 cells were treated with or without 2 mM Omniscan® in the cell culture medium containing 1% CS for 24 h and then characterized by SEM, EDX and FT-IR analysis. (a) SEM image of cells exposed to 1% CS alone for 24 h without treatment with Omniscan®. (b) and (c) Representative SEM images of cells exposed to 2 mM Omniscan® for 24 h. (d) EDX analysis from deposits in a selected area (yellow rectangle) in Fig.1c. (e) FT-IR analysis of the precipitation of 2 mM Omniscan® when added in 1% CS-containing cell culture medium. The black arrows indicated active vibrations of PO_4^{3-} centered at 1066 cm^{-1} and 541 cm^{-1}, respectively.

Omniscan® promoted cell viability via formation of phosphate salt rather than by the compound itself

To clarify whether the Gd chelates themselves or the transformed insoluble Gd-containing deposits formed in the

culture medium promote the cell viability, the effects of filtered or unfiltered Omniscan® medium were compared by filtering the medium through a 220-nm filter. Then, cells were incubated with the unfiltered medium and the filtrates, respectively. As shown in Fig. 2a, 50 μM and 2 mM Omniscan® exerted significant increase in the cell viability while neither of the two filtrates exhibited significant effects. It clearly indicated that the increased cell viability should be mainly attributed to the precipitation of Omniscan® formed in the culture medium, similar to that of $GdCl_3$ [12]. Next, we further compared the effects of other gadolinium-based contrast agents, Magnevist® and Dotarem®, with different thermodynamic stabilities [21]. Both of them with higher chemical stability than Omniscan® but Dotarem® is of the highest stability. As shown in Fig. 2b, 100 μM Magnevist® led to obvious increase of cell viability but Dotarem®, even the dose up to 1 mM, had no obvious effect on 3T3-L1 cells. Meanwhile, the addition of excess ligand diethylenetriaminepentaacetic acid (DTPA) of Omniscan® and Magnevist® could significantly alleviate the increased cell viability induced by the two compounds as seen in Fig.2c. Additionally, FT-IR analysis of the precipitation of Magnevist® formed in the media is similar to that of the omniscan® (data not shown).

The above results demonstrated that the gadolinium chelates such as Omniscan® may form the insoluble phosphates to promote the cell viability under serum-starvation. The Gd chelates with higher thermodynamic stability has the lowest effect. The results also indicated that the compound itself does not appear to promote cell viability under this condition.

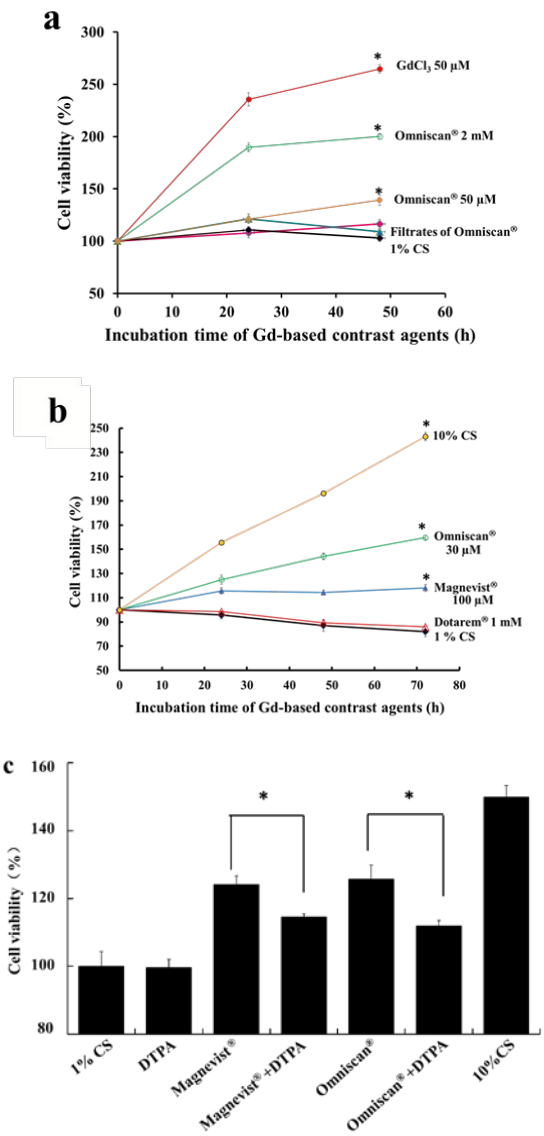
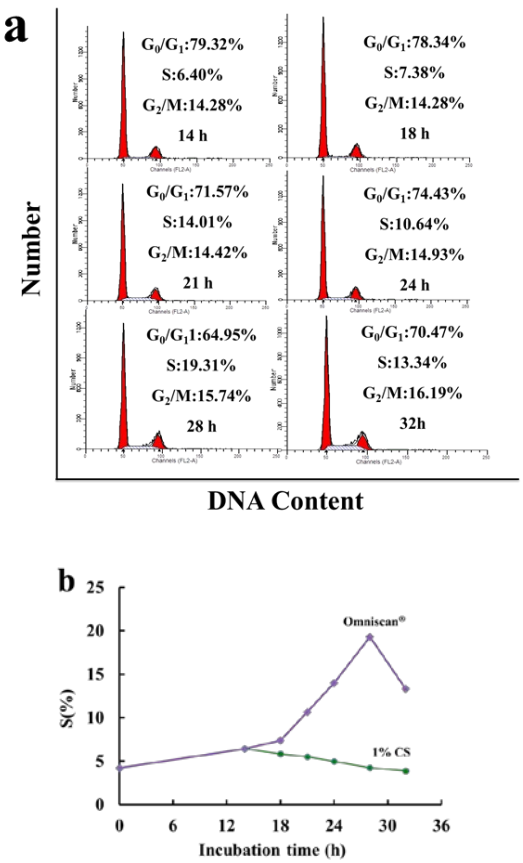


Fig 2. Gadolinium-based contrast agents (GBCAs) Omniscan®, Magnevist® and Dotarem® on 3T3-L1 may form insoluble phosphates to promote the cell viability under serum-starvation. (a) The effects of unfiltered medium and the filtrates of Omniscan® on 3T3-L1 cells viability. The labels in the figure indicated $GdCl_3$ (50 μM), Omniscan® (2 mM and 50 μM), filtrates of different concentrations of Omniscan® (the red one stands for 2 mM and the blue one stands for 50 μM) and 1% CS, respectively. (b) The effect of three gadolinium-based contrast agents Omniscan®, Magnevist® and Dotarem® on 3T3-L1 cells. Cell viability was detected for four consecutive days by MTT assay. (c) The addition of excess ligand DTPA (50 μM) on the cell viability promoted by Magnevist® and Omniscan®. Cells were treated with DTPA, contrast agents or the combination of both for 24 h, respectively. (* $P < 0.05$, significantly different compared with the control group)

Omniscan® promoted G1 to S phase cell cycle progression

To elucidate how Omniscan® increased cell viability after serum starvation, we analysed the cell cycle distribution using flow cytometry. As shown in Fig. 3a and 3b, cells were treated with



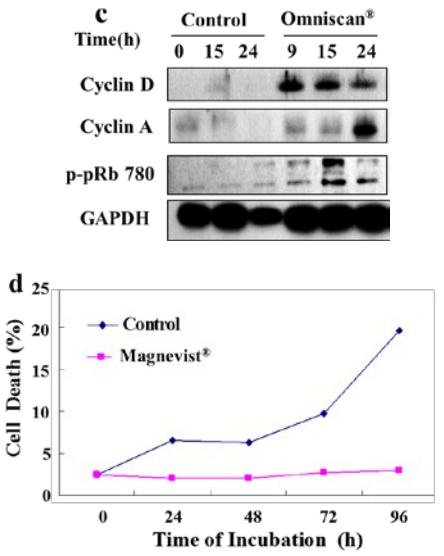


Fig. 3 Omniscan® promoted cell cycle progression in 3T3-L1 cells by enhanced S-phase entry. (a) The distribution of cells in G0/G1, S, and G2/M phases after the treatment with Omniscan® for the indicated time intervals. The hatched areas represent S phase. (b) Quantification of the percentage of cells in S phase after Omniscan® exposure. (c) The expression of cell cycle-associated key proteins after incubation with Omniscan® for various time intervals. (d) Magnevist® alleviated serum starvation-induced cell death.

50 μM Omniscan®, while the control cells were incubated with the DMEM medium containing 1% CS alone. The addition of 50 μM Omniscan® to the cells which were driven into quiescence by serum starvation induced a significant change in the cell cycle distribution. The change was characterized by the increase in S phase starting from 21 h, which was further evidenced by the expression of cell cycle regulatory proteins of G1 to S stage. As seen in Fig. 3c, the level of the cyclins D, cyclins A and the phosphorylated pRb at Ser780 were elevated after the treatment with 50 μM Omniscan® from 9 to 24 h. Thus, it clearly demonstrated that Omniscan® could promote G1 to S phase transition in the serum-starvation 3T3-L1 cells. Although Magnevist® did not significantly promote G1 to S phase cell cycle progression, serum starvation-induced cell death was greatly decreased as shown in Fig.3d. Since accumulation of subG0/G1 cells is usually a characteristic of cells undergoing apoptosis and reflects fragmentation of chromosomal DNA, death fraction was determined as subG0/G1 population measured by flow cytometry [10, 22] . In the absence of Omniscan®, serum starvation led to a sustained increase up to about 20% in the percentage of cell death after 96 h, while with the treatment of Omniscan® death fraction was not significantly changed in consecutive four days, indicating that Omniscan® could prevent serum starvation-induced decrease in cell viability.

Enhanced cell adhesion by gadolinium-based contrast agents was dependent on integrin-mediated signaling

Previously, using live cell station device, we showed that in the presence of $GdCl_3$, cells demonstrated better attachment and became flatten in shapes under the condition of serum-starvation [23]. We also demonstrated that $GdCl_3$-enhanced focal adhesion formation to extracellular matrix played a critical role in cell survival sustaining [10]. Since vinculin is regarded as a universal focal adhesion marker [24, 25], we then investigated the distribution of vinculin to confirm whether Omniscan® and Magnevist® can enhance cell adhesion.

As shown in Fig.4a, we used immunofluorescence to determine whether Omniscan® and Magnevist® can alter the spatial distribution of vinculin. After an additional 18 h serum deprivation as observed previously, many cells were round and

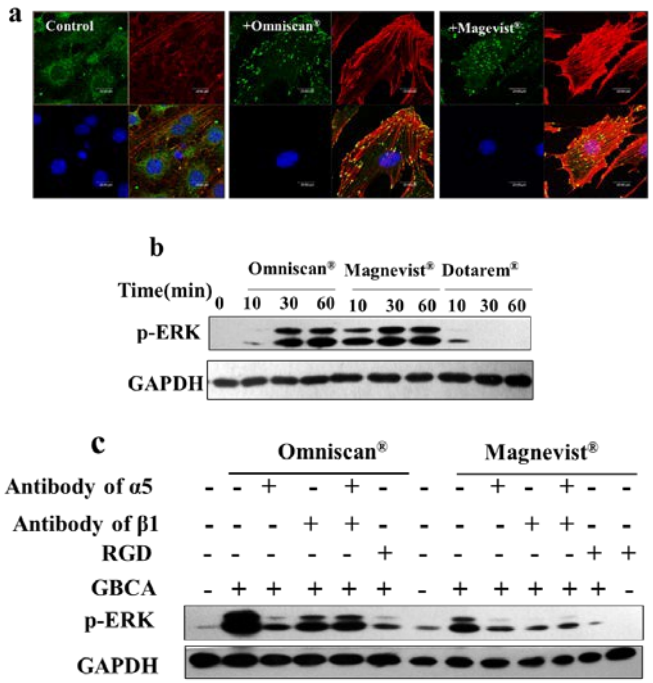


Fig. 4 Gadolinium-based contrast agents promoted cell adhesion to extracellular matrix. The distribution of vinculin (an universal focal adhesion marker) was detected respectively by CLSM after Omniscan®, Magevist® treatment for 18 h. The upper-left area indicates vinculin–fluorescein isothiocyanate stained focal adhesion (green), the upper-right area indicates rhodamine–phalloindin-stained F-actin (red), the lower-left area indicates Hoechst 33258 stained nuclei (blue), and the lower-right area is the overlaid picture. The bars indicate 20 μm. All images shown here are representative immunofluorescence images. (b) The levels of phosphorylated ERK was detected in the presence of 50 μM Omniscan®, Magnevist® or Dotarem®. (c) The effect of blocking antibody of integrin α5 and β1 or RGD on elevated ERK phosphorylation by GBCAs.

the remaining attached cells did not form focal adhesion dots. While in the presence of Omniscan® and Magnevist®, the tendency of detachment was prevented evidenced by the bright dots (green) which indicated the formation of focal adhesions.

Given ERK pathways are necessary for Gd-induced cell cycle progression into S phase [18], we further investigated the ERK activation by the two GBCAs. The result in Fig. 4b revealed that ERK was robustly activated after exposure to 50 μM Omniscan® and Magnevist® for 10 min and its activation was sustained for 60 min while 50 μM Dotarem® had no obvious effects on ERK activation.

To investigate whether integrin-mediated signaling was responsible for Omniscan® and Magnevist®-enhanced cell viability and contributable to ERK activation, cells were pre-

treated with integrin α5 blocking antibody, integrin β1 blocking antibody, or RGD peptide for 1 h. As shown in Fig. 4c, we demonstrated that all of these integrin inhibitors could efficiently alleviate ERK activation induced by Omniscan® and Magnevist®.

Therefore, it can be concluded that Omniscan® and Magnevist®-induced promotion of cell viability was via enhanced cell adhesion and integrin-mediated signaling was involved in this process.

Discussion

We report here that the two Gd-based contrast agents Omniscan® and Magnevist® can convert to insoluble Gd-containing phosphates in the cell culture medium. We also demonstrated that it was the transformed insoluble species rather than Gd chelates themselves that enhance cell survival and promoted S-phase entry in murine 3T3-L1 fibroblasts after serum starvation by filtering the medium through a 220-nm filter. The filtrates of different concentrations of Omniscan® did not induce any significant effect (Fig. 2a). We also found that the three contrast agents differed in the potency of their ability to induce cell viability, which is possibly ascribed to their different thermodynamic stability and further evidenced by the attenuation in cell growth with the addition of excess ligands of the compounds. Omniscan®, as a less thermodynamically stable agent, which was supposed to release more free Gd^{3+} ions than that of Dotarem® and Magnevist®, exerted the highest pro-proliferation effect, which was in consistency with the statistics in clinical practice as evidenced by the fact that Omniscan® has the largest number of reported cases of NSF [26]. Moreover, the integrin-mediated signaling pathway was likely to play an important role in this process by enhancement in focal adhesion formation in a similar way as that of gadolinium chloride [10, 11, 18].

The main reason why Omniscan® share the similar mechanism with gadolinium chloride may be ascribed to that both of them tend to form insoluble particles [3, 15, 16, 27]. The transformation of Omniscan® into insoluble phosphates can be attributed to the higher concentration of phosphates existed in the culture medium [17]. A recent study, using a simplified plasma model, demonstrated that phosphate ion, as the only endogenous ligand that can compete with the ligand of the chelate, bound to the dissociated Gd ion and finally about 17% of the Gd(DTPA-BMA) may form Gd phosphate as a precipitate [27]. Additionally, it has been shown that the elevated serum phosphate levels may not only accelerate the release of Gd^{3+} from nonionic linear GBCAs such as Omniscan® but sensitize renally impaired rats to gadodiamide-induced skin lesions [21, 28]. Indeed, insoluble deposits of Gd have been detected in multiple organs (such as skin, liver, lungs, etc.) of NSF patients and often in fibrotic areas [29-36]. The chemical structure of the subsequently formed insoluble species has been examined to be consistent with a $GdPO_4$ structure [37]. Hyper-phosphatemia was also related to increased risk of NSF for patients with renal failure who received high dose of a GBCA exposure [1, 38]. All of the above evidence indicated that gadolinium phosphate may be as a common mediator for both GBCAs and gadolinium chloride to exert biological effects. The formation of Gd-containing particles may play an important role in the pathophysiology of NSF.

As presented here, if the insoluble deposits are the dorminant working species, it is not surprising that Dotarem® exert the lowest effect over the incubation period since less dissociation of gadolinium ion may resulted in less precipitation. In line with our present results, Fretellier et al [39] compared the Gd concentration of Omniscan® with Dotarem® in renally impaired rats using relaxometry. The results showed dissociation of gadolinium chelates in rats receiving Omniscan®, whereas Dotarem® remained stable over the study period up to 11 days. However, a few of studies also indicated that the intact chelated Gd in the form of Omniscan®, Magnevist®, MultiHance®, and ProHance® increased proliferation of human dermal fibroblasts [12-14]. For example, Bleavins et al [12] compared the proliferative effects among insoluble Gd phosphate, Gd chloride and chelated Gd. They suggested that chelated Gd such as Omniscan® and Magnevist® can also directly stimulate fibroblast survival under low calcium conditions after 3 days exposure. But these studies still cannot exclude the possibility of the converted species of the GBCAs in the medium during the incubation periods ($\geq$ 3 days). Previously, Cabella et al [40] investigated the effects of Gd chelates on HTC and C6 cells. The results showed that 4 h exposure of Gd(DTPA-BMA) (Omniscan®) may cause the net transfer of Gd ions on the cell membrane followed by an internalized process. However, in the absence of phosphate, they found that the internalized Gd content was circa ten times less than that in the presence of phosphate (~1 mM), indicating the formation of Gd phosphate may be responsible for the internalized process although the authors did not state this point clearly. In fact, the previous results have demonstrated that the insoluble Gd phosphate-containing particles can rapidly attached to the fibroblast cells and engulfed into the cells by using scanning and transmission electron microscopy, respectively [11, 12]. Given that the incubation time of Gd chelates in the above studies [12-14] was far more than 4 h, the transformation of Gd chelates cannot be excluded.

As stated above, it is reasonable to speculate that the Gd^{3+} ions dissociated from GBCA such as Omniscan® in biological fluids containing proteins and phosphates, first bound with phosphate and produced the insoluble gadolinium phosphates and followed by depositing on cell surface. The question is how these particles exert the pro-proliferative effect. As we previously proposed, the spontaneously formed Gd-containing particles may act as a reservoir of Gd^{3+} to elicit integrin-mediated signaling pathway [10, 11]. That is to say, even if the concentration of free Gd ion may be very low but once it bound with the active sites of certain membrane receptors, it will be continuously released from the particles to maintain the solubility equilibrium. However, we cannot exclude an alternative possibility that these particles themselves may play a direct role as an intact entity to stimulate certain membrane receptors and triggered downstream signals. We previously reported that Gd-containing particles formed after direct addition of gadolinium chloride into cell culture medium caused cytoskeleton reorganization by live cell imaging system and confocal laser scanning microscopy [23], indicating these

particles may lead to the alteration of phospholipid bilayer membranes by binding with the membrane. The engagement of solid with membrane lipids may subsequently result in the lipid sorting or ion channels activation [41]. Studies with monosodium urate and alum suggested a signaling equivalent platform when cells encounter solid structure in the absence of receptors. The surfaces of these solids can rearrange the membrane lipids and the binding intensity may lead to signaling activation [42, 43]. Thus, we summarize the possible chemical species of GBCAs formed in the cell culture medium and possible ways to trigger pro-survival effect on fibroblasts as indicated in Fig.5.

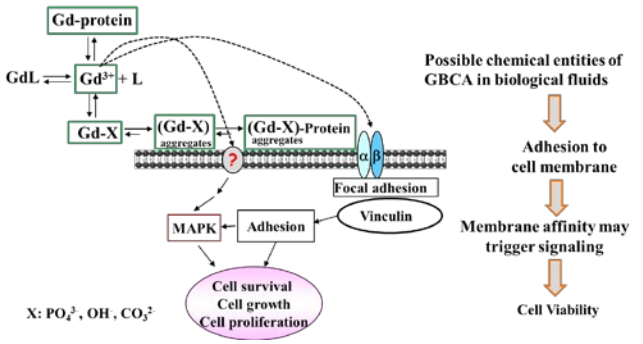


Fig.5 Possible chemical entities of GBCA formed in biological fluids containing proteins and phosphates and possible ways to trigger pro-survival effect on fibroblasts.

Obviously, these chemical species are in a chemical equilibrium thus it is difficult to distinguish the role of each species and the corresponding ways by which these particles exert the biological effect. Further investigations still needed to fully clarify whether the gadolinium phosphates can activate the signaling pathway as an intact entity and the dechelation of GBCAs may act as a priori to lead to the process of NSF.

Conclusion

This study demonstrated that *in situ* precipitation formation mediated gadolinium-based contrast agent Omniscan®-promoted fibroblast survival. The results indicated that GBCAs with high kinetic and thermodynamic stability may minimize the amount of redistribution of Gd by transmetallation and thus may decrease free Gd ions released from tissue accumulation. Our results here supported the notion that GBCAs with more stable thermodynamic stability may lead to lower occurrence of NSF. It may not only help to reveal the pathogenesis of NSF but be helpful for the development of safe gadolinium-based contrast agents.

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Notes and references

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