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Gadolinium-Based Contrast Agents and Free Gadolinium Inhibit Differentiation and Activity of Bone Cell Lineages

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Objectives: Administration of gadolinium-based contrast agents (GBCA) in magnetic resonance imaging results in the long-term retention of gadolinium (Gd) in tissues and organs, including the bone, and may affect their function and metabolism. This study aims to investigate the effects of Gd and GBCA on the proliferation/survival, differentiation, and function of bone cell lineages.

Materials and Methods: Primary murine osteoblasts (OB) and osteoclast progenitor cells (OPC) isolated from *C57BL/6J* mice were used to test the effects of Gd^{3+} (12.5–100 μM) and GBCA (100–2000 μM). Cultures were supplemented with the nonionic linear Gd-DTPA-BMA (gadodiamide), ionic linear Gd-DTPA (gadopentetic acid), and macrocyclic Gd-DOTA (gadoteric acid). Cell viability and differentiation were analyzed on days 4–6 of the culture. To assess the resorptive activity of osteoclasts, the cells were grown in OPC cultures and were seeded onto layers of amorphous calcium phosphate with incorporated Gd.

Results: Gd^{3+} did not affect OB viability, but differentiation was reduced dose-dependently up to 72.4% ± 6.2%–73.0% ± 13.2% (average ± SD) at 100 μM Gd^{3+} on days 4–6 of culture as compared with unexposed controls ($P < 0.001$). Exposure to GBCA had minor effects on OB viability with a dose-dependent reduction up to 23.3% ± 10.2% for Gd-DTPA-BMA at 2000 μM on day 5 ($P < 0.001$). In contrast, all 3 GBCA caused a dose-dependent reduction of differentiation up to 88.3% ± 5.2% for Gd-DTPA-BMA, 49.8% ± 16.0% for Gd-DTPA, and 23.1% ± 8.7% for Gd-DOTA at 2000 μM on day 5 ($P < 0.001$). In cultures of OPC, cell viability was not affected by Gd^{3+}, whereas differentiation was decreased by 45.3% ± 9.8%–48.5% ± 15.8% at 100 μM Gd^{3+} on days 4–6 ($P < 0.05$). Exposure of OPC to GBCA resulted in a dose-dependent increase in cell viability of up to 34.1% ± 11.4% at 2000 μM on day 5 of culture ($P < 0.001$). However, differentiation of OPC cultures was reduced on day 5 by 24.2% ± 9.4% for Gd-DTPA-BMA, 47.1% ± 14.0% for Gd-DTPA, and 38.2% ± 10.0% for Gd-DOTA ($P < 0.001$). The dissolution of amorphous calcium phosphate by mature osteoclasts was reduced by 36.3% ± 5.3% upon incorporation of 4.3% Gd/Ca wt/wt ($P < 0.001$).

Conclusions: Gadolinium and GBCA inhibit differentiation and activity of bone cell lineages in vitro. Thus, Gd retention in bone tissue could potentially impair the physiological regulation of bone turnover on a cellular level, leading to pathological changes in bone metabolism.

Key Words: gadolinium retention, gadolinium, contrast agent, murine bone cells, in vitro, cell culture

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Over the past 30 years, nearly 500 million doses of gadolinium-based contrast agents (GBCA) have been administered worldwide in diagnostic radiology.[1] Gadolinium-based contrast agent Gadolinium-based contrast agents, in which Gd^{3+} is bound to a chelator to decrease its toxicity, can be assigned to 4 groups that are characterized by their structural (linear/macrocyclic) and electrochemical (ionic/nonionic) properties. The structure and charge of these chelators determine their pharmacodynamic and pharmacokinetic properties.[2]

In patients with impaired renal function, the extended presence of GBCA in the circulation leads to Gd retention in the skin and other organs, which may lead to nephrogenic systemic fibrosis.[3,4] In particular, nonionic linear and ionic linear GBCA, including Gd-DTPA-BMA (gadodiamide) and Gd-DTPA (gadopentetic acid), respectively, were associated with this potentially life-threatening disease.[3,5] The decreased renal clearance, in combination with the relative instability of linear contrast agents, led to prolonged persistence of the GBCA within the human body and to a higher dissociation rate. These severe adverse effects are now virtually eliminated by reducing the minimum necessary dose of the GBCA applied and by using preferentially macrocyclic contrast agents.[6] Recent studies documented that Gd is retained also in patients with normal renal function after single or multiple applications of all commercially available GBCA in the central nervous system, skeleton, and in organs involved in drug elimination, such as liver, spleen, and kidney, the level of retention depending on the specific agent.[7,8] Presently, in patients with normal kidney function, retention of Gd is not linked to clinically relevant adverse events,[9] and the clinical recognition of a Gd deposition disease is still controversial.[10] Although a causal link between Gd deposition and clinical manifestations remains uncertain,[11] real-life safety data demonstrate that the use of linear agents, particularly Gd-BOPTA, and, to some extent, the macrocyclic Gd-HP-DO3A, increased the occurrence of symptoms associated with Gd exposure.[12]

Consequently, the observed Gd retention raises concerns regarding the safety of GBCA, particularly after frequent use, but the form of Gd that is deposited in biological tissues remains unclear. The GBCA complex may remain intact or dissociate,[13] the latter of which seems most likely to occur in the bone.[14,15] Recent studies in preclinical rat models developed a novel technique to differentiate between chelated and dechelated Gd in brain[16] and bone[15] tissue. Released Gd^{3+} will most likely be rechelated by low-molecular-weight ligands, such as citrate or lactate, will bind to macromolecules or proteins,[15,16] or will form insoluble salts with phosphate or carbonate[16,17] and thus may be incorporated into the inorganic bone matrix.[18] Gadolinium retained in bone tissue was detected after administration of every type of contrast agent.[19,20] Nevertheless, in particular, linear contrast agents resulted in higher amounts of retained Gd, which was confirmed in clinical[7,8,21,22] and preclinical[15,16,23] studies.

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In the bone of femoral heads, Gd deposition was discovered even 8 years after the application of linear and macrocyclic GBCA.[24] The amount of Gd measured per kilogram of bone was found to be 0.1–5 g (0.1–5 g/kg; 0.62–32 μmol/kg),[7,19,20] which exceeds the Gd found in neuronal structures by 10–1000 fold.[22,25] Based on these observations, it was hypothesized that Gd retained in the skeleton may affect bone metabolism when it is released during bone turnover. A recent study on Gd deposition in rat bones reported that Gd was initially located at external bone sites close to the periosteum and bone cavities. After 1 year, the newly formed bone covers the Gd-containing layer.[26] However, the entire adult skeleton is completely replaced within approximately 10 years, as 5%–10% of the bone tissue is remodeled annually.[27] Hence, Gd incorporated into deeper layers of the bone tissue will be released during bone modeling and remodeling.

In consideration of the frequent use of GBCA, further investigations are justified to understand the impact of Gd deposition in bone tissues. Therefore, this study focused on elucidating the effects of Gd^{3+} and GBCA on the differentiation, proliferation, and function of bone lineage cells.

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MATERIALS AND METHODS

Animals

Animal experiments were performed in accordance with the Swiss Federal regulations and approved by the Cantonal Veterinary Office (permit numbers: BE 19/19 and BE29/2022 to W.H.). Animals were maintained under specific pathogen-free conditions at the Animal Facility of the University of Bern.

Culture of Osteoblasts

Primary osteoblasts (OB) were isolated from the calvariae of 1- to 2-day-old *C57BL/6J* mice through sequential collagenase type I digestion[28] and stored in liquid nitrogen at 1×10^6 cells/mL. For each experiment, cells were thawed and cultured in medium containing α-MEM (Gibco; LuBio Science, Lucerne, Switzerland), 10% heat-inactivated fetal bovine serum (FBS), and 1% penicillin-streptomycin (Gibco; LuBio Science). After 4 days of expansion, OB were replated in 96-well plates (20,000 cells/mL, 0.1 mL) with medium containing 10% FBS and 1% penicillin-streptomycin. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was changed after 72 hours. To induce cell differentiation of OB cultures, media were supplemented with bone morphogenetic protein-2 (64 nM; Department of Physiological Chemistry, University of Würzburg, Würzburg, Germany) and 1,25$(OH)_2$ vitamin D (10 nM). To study the effects of free Gd^{3+}, culture media were supplemented with 12.5–100 μM Gd^{3+} (gadolinium [III]-chloride hexahydrate). This concentration range was selected, as 100 μM of free Gd^{3+} did not yet affect cell viability but allowed for the efficient evaluation of cell differentiation. To study the effects of chelated Gd^{3+}, culture media were supplemented with 3 representative compounds of different GBCA classes, namely, Gd-DTPA-BMA (gadodiamide, Omniscan; GE HealthCare, Chicago, IL), Gd-DTPA (gadopentetic acid, Magnevist; Bayer, Berlin, Germany), and Gd-DOTA (gadoteric acid, Dotarem; Guerbet, Villepinte, France). Considering the release rate of approximately 2.6%–4.5% for the nonionic linear Gd-DTPA-BMA,[13] a concentration range of 100–2000 μM was used in these experiments, as 2000 μM Gd-DTPA-BMA will result in 50–100 μM of free Gd^{3+} after 3 days of culture. To investigate the impact of empty chelators on OB cultures, cell culture media were supplemented with 100 μM DTPA (pentetic acid) or DOTA (dodecane tetraacetic acid) with or without addition of increasing concentrations of Gd^{3+} (12.5–100 μM). All reagents were purchased from Sigma-Aldrich (St Louis, MO) if not stated otherwise.

Culture of Osteoclast Progenitor Cells

Bone marrow cells were isolated from long bones of *C57BL/6J* mice (5–16 weeks) and cultured in medium supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin, and 30 ng/mL colony-stimulating factor 1 (CSF-1; FDP-Chiron, Emeryville, CA). After 24 hours, nonadherent osteoclast progenitor cells (OPC) were replated in 96-well plates (200,000 cells/mL, 0.1 mL) in medium supplemented with 10% FBS, 1% penicillin-streptomycin, 30 ng/mL CSF-1, and 20 ng/mL receptor activator of nuclear factor-κB ligand (RANKL; PeproTech; LuBio Science) to induce osteoclastogenesis. The media were supplemented with Gd^{3+} (12.5–100 μM) or GBCA (100–1000 μM) and changed after 72 hours.

Determination of Cell Viability (XTT Assay)

Cell viability was assessed at days 4, 5, and 6 of the cultures using a Cell Proliferation Kit II (XTT; Roche Diagnostics, Basel, Switzerland). Briefly, OB or OPC cultures were incubated with XTT tetrazolium salt for 3 hours and 4 hours, respectively. The absorbance was measured at 470 nm using a Spark Multimode Spectrophotometer (Tecan Group Ltd, Männedorf, Switzerland) with a reference wavelength of 630 nm.

Assessment of OB Differentiation

Osteoblast differentiation was assessed by alkaline phosphatase (ALP) activity, a marker for OB differentiation, on days 4–6 of culture as described previously.[29] Cultures in 96-well plates were washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, and 12 mM Pi, pH 7.4), lysed with 25 μL 0.1% Triton X-100, and frozen at −20°C. Subsequently, 25 μL of ALP substrate (4-nitrophenyl phosphate, 1 mg/mL), dissolved in diethanolamine buffer (1 M diethanolamine, pH 9.8), was added. The enzymatic reaction was terminated after 15–30 minutes with 0.1 M EDTA, and the absorbance was measured at 405 nm and at the reference wavelength of 630 nm. Alkaline phosphatase results were normalized against cell viability.

Assessment of OPC Differentiation

Tartrate-resistant acid phosphatase (TRAP) activity, a marker of osteoclast (OC) maturation, was measured on days 4–6 of OPC culture.[30] Cultures in 96-well plates were washed with PBS and lysed with 50 μL 0.1% Triton X-100 solved in 1 M NaCl solution and frozen at −20°C. Subsequently, TRAP substrate (4-nitrophenyl phosphate, 4.61 mg/mL) dissolved in 40 mM sodium tartrate and 50 mM sodium acetate, pH 4.8, was added, and the enzymatic reaction was terminated after 1 hour with 0.2 N NaOH. Absorbance was measured at 405 nm and at the reference wavelength of 630 nm.

In addition, OC were visualized using a commercial TRAP kit (Sigma-Aldrich). After fixation with 4% paraformaldehyde for 10 minutes at room temperature, cells were incubated with TRAP staining solution (5 minutes). Mature OC were counted as multinucleated TRAP-positive cells (nuclei count >3).

Assessment of OC Activity

To assess the capacity of OC to dissolve amorphous calcium phosphate (CaP), mature OC were plated onto CaP layers spiked with ^{45}Ca, as previously described.[30] The amorphous CaP was generated by mixing equal volumes of 0.12 M Na_2HPO_4 and 0.2 M $CaCl_2$. The $CaCl_2$ solution was spiked with ^{45}Ca (200 MBq/mL; Perkin Elmer, Schwerzenbach, Switzerland) and Gd^{3+} to reach a final concentration of 100 μM Gd in the amorphous CaP slurry. The precipitated CaP slurry was rinsed twice with sterile distilled water and further diluted with additional water to a concentration of 50 μg/mL. To produce the CaP layers, 200 μL/well of the suspension (3500 Bq ^{45}Ca) were transferred into 48-well plates, dried (72 hours, room temperature), and baked for 3 hours at 80°C. The final amount of Gd in the dried CaP layers was quantified by inductively coupled plasma mass spectrometry, resulting in 4.3% Gd/Ca wt/wt (Supplemental Digital Content 1, Table S1, http://links.lww.com/RLI/A886). Approximately 95% of the added Gd^{3+} was incorporated when comparing Gd input to Gd incorporation into CaP layers. The CaP layers were stable under cell culture conditions without spontaneous release of Gd.

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Mature OC were generated by culturing OPC (150,000 cells/mL, 5 mL, 20 cm^2) in culture medium supplemented with 10% FBS, 1% penicillin-streptomycin, CSF-1 (30 ng/mL), and RANKL (20 ng/mL). After 5 days, OC were released from the culture dish with 0.02% EDTA in PBS (37°C, 15 minutes). Detached cells were centrifuged and resuspended in medium (500 μL/20 cm^2 dish). Subsequently, OC (50 μL suspension/well) were transferred onto the CaP layers with medium (200 μL/well) supplemented with HCl (15 mM), CSF-1 (30 ng/mL), and RANKL (20 ng/mL). Osteoclasts exposed to calcitonin (10 nM, salmon-derived) were used as negative controls.

Release of ^{45}Ca into the medium was measured after 24 hours using a scintillation counter (Tri-Carb 2300 TR Liquid Scintillation Analyzer; Packard—A Canberra Company). Cells were subsequently lysed to assess TRAP activity. The amount of radioactivity in the culture medium, normalized to TRAP, was used as an indicator of the mineral dissolution capacity of OC. To visualize OC, cells were fixed with 4% paraformaldehyde and stained with TRAP solution, as described previously.[31] Resorption pits in the CaP layers were stained using the Von Kossa protocol.[32]

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Statistical Analysis

Statistical analyses were performed using GraphPad Prism 10 (GraphPad Software, San Diego, CA). Kruskal-Wallis test with Dunn multiple comparison test was used to detect statistically significant differences. Differences were termed statistically significant with values of $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$.

RESULTS

Exposure to Gd^{3+} or GBCA Inhibits Cell Differentiation of OB Cultures

To investigate potential effects of Gd^{3+} and GBCA on OB lineage cells, primary murine OB were cultured in media supplemented with Gd^{3+} (12.5–100 μM). Gd^{3+} did not affect OB viability after 4–6 days in culture (Fig. 1A). Differentiation, as assessed by ALP activity, was dose-dependently reduced at all tested concentrations compared with control cells (Fig. 1B). Upon normalization for cell viability, ALP activity was reduced on days 4–6 by up to 72.4% ± 6.2%–73.0% ± 13.2% at 100 μM Gd^{3+} ($P < 0.001$) compared with control cells (Fig. 1C).

To test the biological effects of GBCA, media were supplemented with 3 selected contrast agents (100–2000 μM). Gadolinium-based contrast agents had minor impact on OB viability after 5 days in culture (Fig. 2A). Exposure to Gd-DTPA-BMA led to a reduction of OB viability up to 23.3% ± 10.2% ($P < 0.001$) at the highest concentration tested (Fig. 2A). In contrast, differentiation was inhibited in a dose-dependent manner by each of the tested agents (Fig. 2B). Exposure of OB cultures to nonionic linear Gd-DTPA-BMA reduced the ALP activity normalized

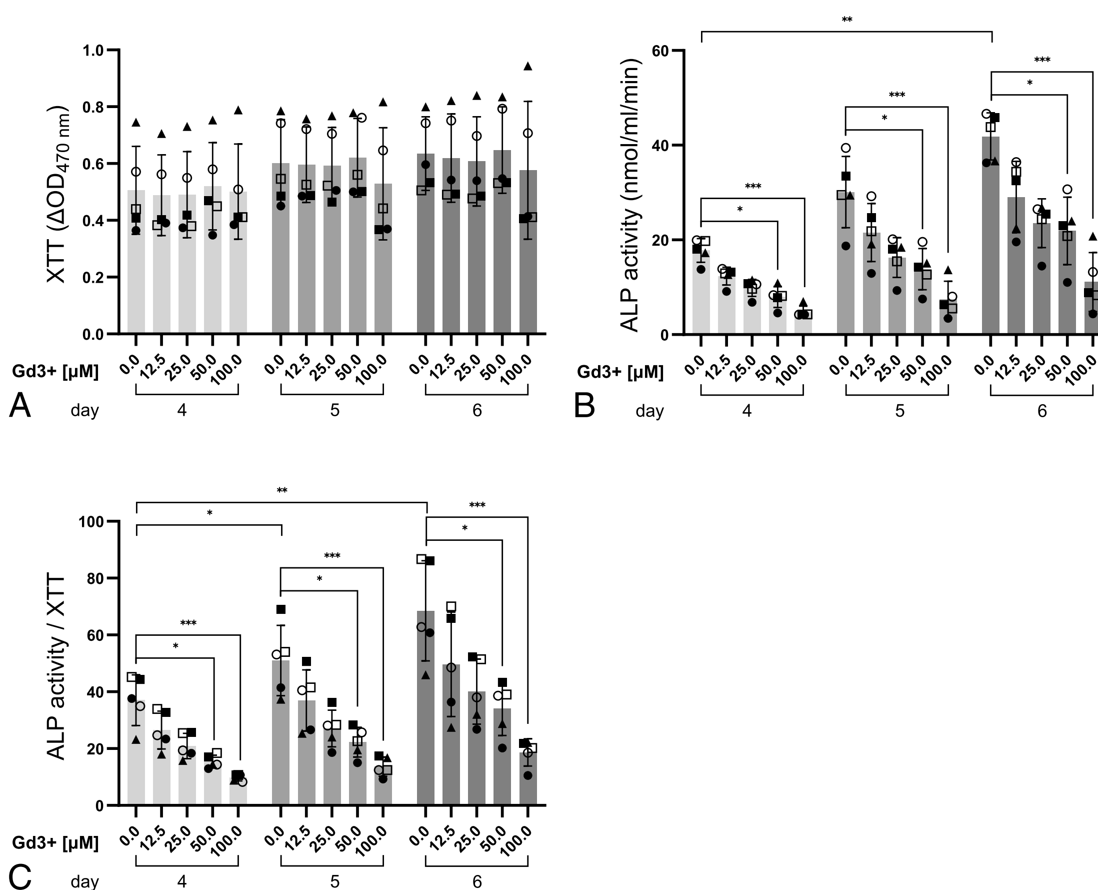

FIGURE 1. Effect of Gd^{3+} on the viability and differentiation of OB. Effect of Gd^{3+} (12.5–100 μM) on OB viability (A), ALP activity (B), and ALP activity normalized to cell viability (C) on days 4, 5, and 6 of culture. To stimulate OB differentiation, cultures were supplemented with bone morphogenetic protein-2 (64 nM) and 1,25$(OH)_2$ vitamin D (10 nM). Statistical differences were determined using the Kruskal-Wallis test with Dunn test by comparing to the control group (0 μM). Differences between culture days were calculated for the control groups (0 μM) comparing to the control at day 4. Differences were termed statistically significant with values of $P \leq 0.05$ (*), $P \leq 0.01$ (**), and $P \leq 0.001$ (***). Data are presented as the mean ± standard deviation with 5 biological replicates (n = 5) in technical replicates of 6. Biological replicates are depicted with different symbols. OB, osteoblast; ALP, alkaline phosphatase.

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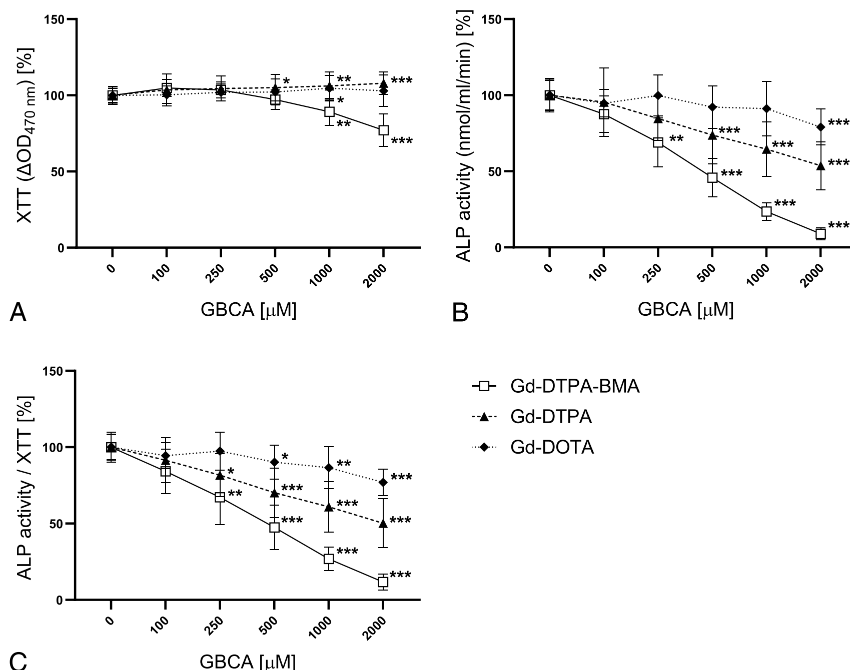

FIGURE 2. Effect of GBCA on the viability and differentiation of OB. Effect of Gd-DTPA-BMA, Gd-DTPA, and Gd-DOTA (100–2000 μM) on OB viability (A), ALP activity (B), and ALP activity normalized to cell viability (C) on day 5 of culture. To stimulate OB differentiation, cultures were supplemented with bone morphogenetic protein-2 (64 nM) and 1,25(OH)$_2$ vitamin D (10 nM). Values are normalized to controls (0 μM Gd^{3+}) and displayed in % (control = 100%). Statistical differences were determined using the Kruskal-Wallis test with Dunn test by comparing to the control group (0 μM). Differences were termed statistically significant with values of $P \leq 0.05$ (*), $P \leq 0.01$ (**), and $P \leq 0.001$ (***). Data are presented as the mean ± standard deviation in biological replicates of 3–4 (n = 3–4) in technical replicates of 6. GBCA, gadolinium-based contrast agent; OB, osteoblast; ALP, alkaline phosphatase.

for cell viability (Fig. 2C) by 23.3% ± 17.8% at 250 μM ($P < 0.01$) and 88.3% ± 5.2% at 2000 μM ($P < 0.001$); ionic-linear Gd-DTPA reduced the normalized ALP activity by 18.3% ± 14.0% at 250 μM ($P < 0.01$) and 49.8% ± 16.0% at 2000 μM ($P < 0.01$); lastly, macrocyclic Gd-DOTA reduced the normalized ALP activity by 9.9% ± 11.2% at 500 μM ($P < 0.05$) and 23.1% ± 8.7% at 2000 μM ($P < 0.001$). Similar results were found after 4 and 6 days of culture (Supplemental Digital Content 1, Fig. S1, http://links.lww.com/RLI/A886). Overall, exposure of OB to Gd^{3+} and GBCA inhibited cell differentiation in a dose-dependent manner, as assessed by ALP activity, with only minor effects on cell viability.

Exposure to the Chelator DTPA Inhibits Cell Proliferation and Differentiation of Cultures of Primary OB

After clinical application, small amounts of Gd^{3+} dissociate from GBCA.[13] It remains unclear whether eventual cellular effects are caused by complexed or free Gd^{3+} or by chelator molecules. Hence, OB cultures were exposed to DTPA and DOTA alone or in combination with Gd^{3+} (Fig. 3; Supplemental Digital Content 1, Table S2, http://links.lww.com/RLI/A886). XTT levels and ALP activity were reduced by 27.5% ± 17.2% and 80.5% ± 5.6% ($P < 0.001$), respectively, in cultures supplemented with 100 μM of the ionic linear chelator DTPA. In contrast, the macrocyclic chelator DOTA (100 μM) did not affect OB proliferation or differentiation. XTT levels and ALP activity were reduced by 17.8% ± 23.8% ($P < 0.05$) and 45.9% ± 7.1% ($P < 0.001$), respectively, in cultures supplemented with 100 μM DTPA and 12.5 μM Gd^{3+} compared with cells treated with 12.5 μM Gd^{3+} alone. DTPA (100 μM) together with 25 μM Gd^{3+} reduced ALP activity by 29.2% ± 10.4% compared with cells treated with 25 μM Gd^{3+} alone ($P < 0.001$). Equimolar amounts of Gd^{3+} and DTPA increased OB viability by 25.2% ± 21.1% and ALP activity by 68.0% ± 16.8% compared with that by 100 μM Gd^{3+} ($P < 0.001$). DOTA blocked the inhibitory effect of 100 μM Gd^{3+} on OB proliferation (XTT increase of 33.4% ± 26.2%, $P < 0.001$). In addition, the negative impact of Gd^{3+} (12.5–100 μM) on OB differentiation was neutralized by DOTA and resulted in increased ALP activity by approximately 30% to 60% compared with cells treated with corresponding Gd^{3+} concentrations ($P < 0.01$). Similar results were found after 4 and 6 days of culture (Supplemental Digital Content 1, Fig. S2, http://links.lww.com/RLI/A886). These findings demonstrated that only the ionic linear chelator DTPA caused a reduction in OB proliferation and differentiation. Both DTPA and DOTA reversed the negative effects of Gd^{3+} on OB cultures.

Exposure to Gd^{3+} or GBCA Increases Cell Viability of OPC/OC Cultures and Is Accompanied by Partial Inhibition of Cell Differentiation

Bone turnover encompasses the coordinated activation of OB and OC lineage cells. Therefore, the effects of Gd^{3+} and GBCA on OC development and activity were investigated to elucidate their potential effects on bone resorption.

Exogenously added Gd^{3+} did not affect OPC viability (Fig. 4A) on days 4 and 5 of culture. On day 6, untreated control cells showed an approximately 50% decrease in cell viability compared with day 4 ($P < 0.01$), whereas XTT levels in cultures of OPC exposed to 100 μM Gd^{3+} remained stable between days 4 and 6. Tartrate-resistant acid phosphatase activity normalized for cell viability (Fig. 4C), a parameter for osteoclastic differentiation, was reduced in a dose-dependent manner on days 4 and 5, reaching significance only with 100 μM Gd^{3+} ($P < 0.05$). On day 6, Gd^{3+} supplementation caused a decline of TRAP activity of 36.1% ± 10.9% for 50 μM ($P < 0.01$) and 45.3% ± 9.8% for 100 μM ($P < 0.001$). Staining of TRAP-positive cells showed a dose-dependent decrease in the number of mature OC on day 4 by 32.1% ± 6.8% at 50 μM Gd^{3+} and 73.7% ± 14.0% at 100 μM Gd^{3+} ($P < 0.05$) (Fig. 4D). On days 5 and 6, the reduction of TRAP-positive cells was not significant.

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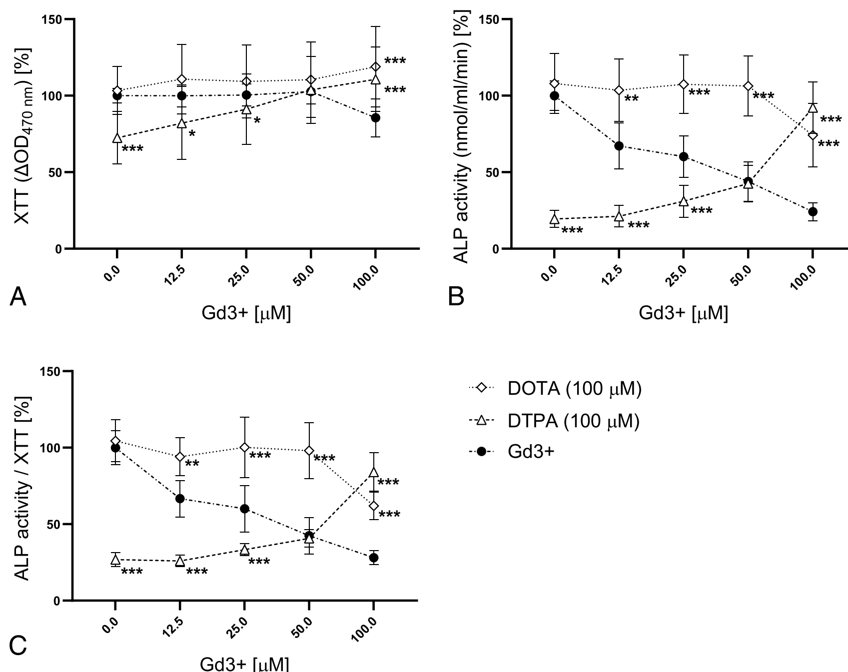

FIGURE 3. Effect of the free chelators DTPA and DOTA on OB viability and differentiation (day 5). OB cultures were supplemented with increasing concentration of Gd3+ (0–100 μM) in combination with 100 μM of the empty chelator DTPA or DOTA. The effect of empty chelators on OB viability (A), ALP activity (B), and ALP activity normalized on cell viability (C) on day 5. To stimulate OB differentiation, cultures were supplemented with bone morphogenetic protein-2 (64 nM) and 1,25(OH)$_2$ vitamin D (10 nM). Values are normalized to controls (0 μM Gd^{3+}) and displayed in % (control = 100%). Statistical differences were determined using the Kruskal-Wallis test with Dunn test by comparing to the Gd^{3+} culture. Differences were termed statistically significant with values of $P \leq 0.05$ (*), $P \leq 0.01$ (**), and $P \leq 0.001$ (***). Data are presented as the mean ± standard deviation with 4 biological replicates (n = 4) in technical replicates of 6. OB, osteoblast; ALP, alkaline phosphatase.

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Exposure of OPC to GBCA led to a dose-dependent rise in cell viability on day 5 of culture (Fig. 5A) with an average increase of 34.1% ± 11.4% at 2000 μM ($P < 0.001$). Cell differentiation, as assessed by normalized TRAP activity, was inhibited in a dose-dependent manner by the tested agents (Fig. 5C). Exposure to 2000 μM GBCA decreased the normalized TRAP levels by 24.2% ± 9.4% with Gd-DTPA-BMA, 47.1% ± 14.0% with Gd-DTPA, and 38.2% ± 10.0% with Gd-DOTA ($P \leq 0.001$). Incubation of OPC cultures with 2000 μM GBCA led to a significant decrease in the viability of multinucleated OC, with an average decrease of 34.6% ± 9.6% ($P < 0.001$) (Fig. 5D). Similar results were found after 4 and 6 days of culture (Supplemental Digital Content 1, Fig. S3, http://links.lww.com/RLI/A886), with similar results. In summary, OPC exposed to Gd^{3+} or GBCA showed a dose-dependent increase in cell viability and concomitant inhibition of cell differentiation.

The Activity of Mature OC is Reduced by Gd Incorporated into Amorphous CaP

To mimic the effects of Gd incorporated into bone tissue on the resorptive activity of OC, amorphous CaP layers spiked with ^{45}Ca and enriched with Gd were generated (Fig. 6A). Dissolution of CaP containing 4.3% Gd/Ca wt/wt by mature OC was reduced by 36.3% ± 5.3% ($P < 0.001$) compared with CaP without Gd. Von Kossa staining indicated that OC formed smaller resorption lacunae when dissolving the Gd-CaP layers (Fig. 6B).

DISCUSSION

Gadolinium-based contrast agents are routinely used in diagnostic radiology. Their clinical application may be complicated by the retention of Gd in various organs, including the brain, skin, and bones. It has been hypothesized that the bone functions as a long-term reservoir for Gd,[22] and the continuous release of Gd or chelators during modeling and remodeling processes may impact the metabolism of the skeleton and other organ systems. To characterize potential adverse effects of Gd on cell and tissue functions, this study describes the effects of Gd^{3+} and GBCA on the survival and function of bone cell lineages.

Primary murine OB and OPC cultures were chosen as in vitro model systems to study the influence of free and complexed Gd^{3+}. Since it is virtually not possible to assess the relevant in vivo concentrations of Gd^{3+} and GBCA, different concentrations of the compounds, each in a dose curve, were tested. The data need to be considered as a proof of principle, and further in vivo and clinical studies will be required to assess the effects of the compounds on bone metabolism under physiological conditions. The concentration range of 12.5–100 μM of free Gd^{3+} did not affect cell viability but allowed for the evaluation of cell differentiation. In a previous study, the stability of GBCA in human serum was investigated, and a release of Gd^{3+} ranging from 2.6% to 4.5% for the nonionic linear Gd-DTPA-BMA after 3 days was found in cell culture conditions.[13] Considering this release rate, a concentration range of 100–2000 μM was defined for the tested GBCA, as 2000 μM Gd-DTPA-BMA may potentially result in 50–100 μM of free Gd^{3+}. After testing 7 contrast agents, representing the 4 classes of GBCA, in OB and OPC/OC cultures, the compounds Gd-DOTA, Gd-DTPA, and Gd-DTPA-BMA were selected for further experiments. The effects of the nonionic linear Gd-DTPA-BMA differed from the effects of the ionic linear contrast agents (Gd-DTPA, Gd-EOB-DTPA, and Gd-BOPTA), which as a group exerted comparable effects on the viability and differentiation of the cell cultures. In contrast, macrocyclic GBCA, both nonionic (Gd-DO3A-butrol, Gd-HP-DO3A) and ionic (Gd-DOTA), showed similar

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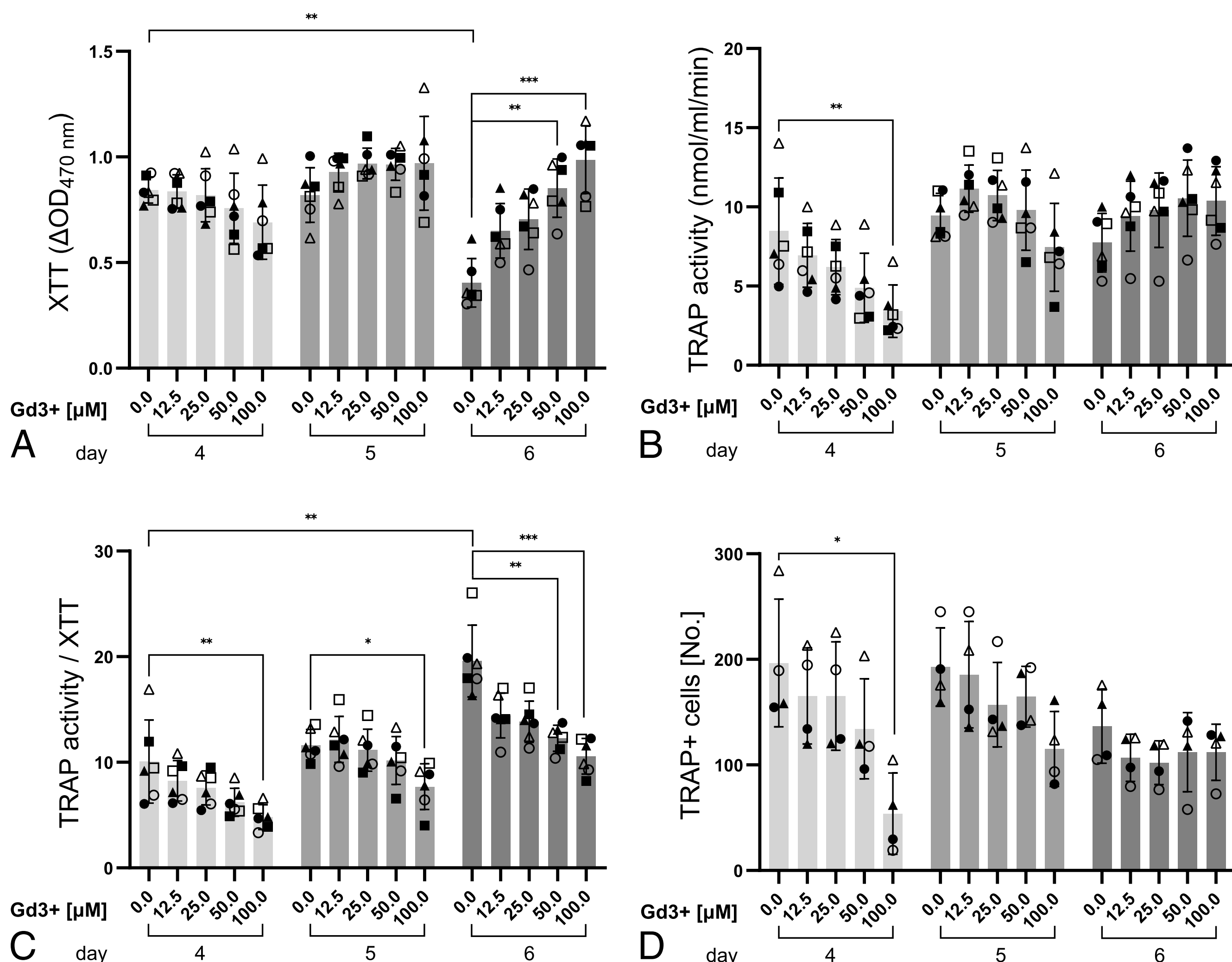

FIGURE 4. Effect of Gd^{3+} on OPC/OC viability and differentiation. Effect of Gd^{3+} (12.5–100 μM) on OPC/OC viability (A), TRAP activity (B), TRAP activity normalized to cell viability (C), and viability of TRAP-positive multinucleated cells (D) on days 4, 5, and 6. OPC/OC cultures were supplemented with CSF-1 (30 ng/mL) and RANKL (20 ng/mL) to stimulate OPC/OC development and maturation. Statistical differences were determined using the Kruskal-Wallis test with Dunn test by comparing to the control group (0 μM). Differences between culture days were calculated for the control groups (0 μM) comparing to the control at day 4. Differences were termed statistically significant with values of $P \le 0.05$ (*), $P \le 0.01$ (**), and $P \le 0.001$ (***). Data are presented as the mean ± standard deviation with 4–6 biological replicates (n = 4–6) in technical replicates of 6. Biological replicates are depicted with different symbols. OPC, osteoclast progenitor cell; OC, osteoclast; TRAP, tartrate-resistant acid phosphatase; CSF-1, colony-stimulating factor 1; RANKL, receptor activator of nuclear factor-κB ligand.

effects on OB and OPC/OC cultures, and Gd-DOTA was chosen as the representative compound.

The exposure to Gd^{3+} and GBCA caused a dose-dependent inhibition of the differentiation of primary murine OB but did not or only slightly affect cell viability. The degree of inhibition of osteoblastic differentiation (Gd-DTPA-BMA > Gd-DTPA > Gd-DOTA) correlated with the thermodynamic stability of the respective contrast agents. This suggests that the effects of GBCA on OB cultures are at least partially caused by free Gd^{3+} dissociated from the complex and possibly to some extent by empty chelator molecules.

Gd^{3+} has no physiological role in the organism but can compete with calcium ions (Ca^{2+}) due to their similar atomic radii[33] and may therefore interfere with cellular functions by acting as a Ca^{2+} antagonist. A possible mechanism explaining the inhibition of OB differentiation by Gd^{3+} is the interruption of the bone morphogenetic protein-2–mediated increase in intracellular Ca^{2+} by blocking the influx of extracellular Ca^{2+} and its release from intracellular stores.[34,35] Moreover, Ca^{2+} signaling modulates OB differentiation and activity,[36] which may be antagonized by free Gd^{3+}. In addition, studies provided evidence that Gd^{3+} inhibits Ca^{2+}-mediated signaling in OB lineage cells after mechanostimulation or fluid shear stress by blocking Gd^{3+}-sensitive Ca^{2+} entry channels,[37,38] a mechanism not attributable to the current cell culture system.

Gadolinium-based contrast agents dissociate partially after administration during diagnostic radiology.[39,40] Therefore, supplementing culture media with empty chelator molecules will allow to analyze their potential effects on the functions of bone cell lineages. Cell viability and differentiation in OB were reduced in cultures treated with the ionic linear chelator DTPA. Upon addition of increasing amounts of Gd^{3+}, the negative effects of the chelator and Gd^{3+} were gradually neutralized. In contrast, the macrocyclic chelator DOTA blocked the inhibitory effects of Gd^{3+} on OB without exerting any cellular effects by itself. It

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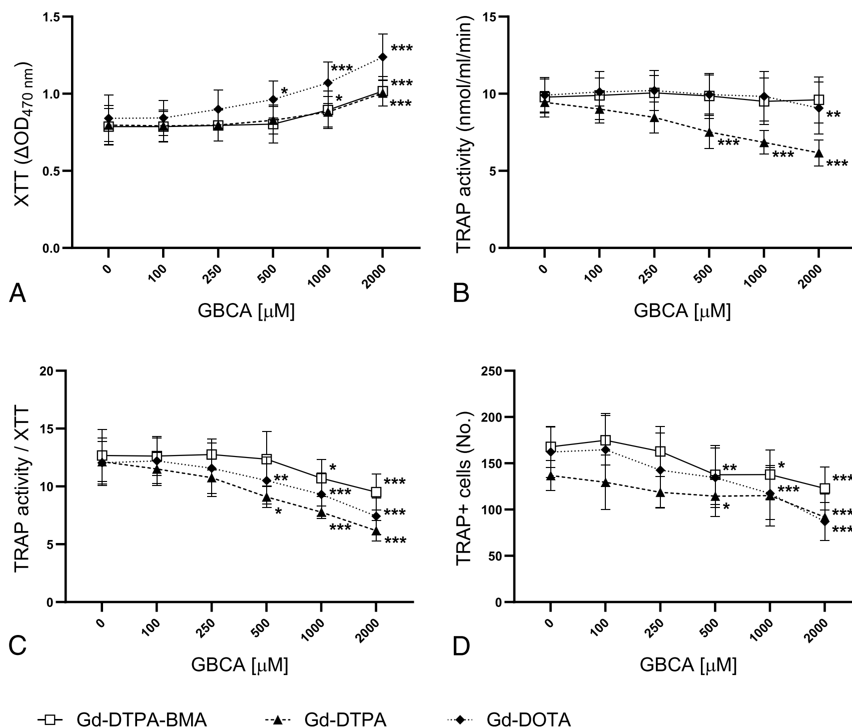

FIGURE 5. Effect of GBCA on OPC/OC viability and differentiation. Effect of Gd-DTPA-BMA, Gd-DTPA, and Gd-DOTA (100–2000 μM) on OPC/OC viability (A), TRAP activity (B), TRAP activity normalized to cell viability (C), and number of TRAP-positive multinucleated cells (D) on day 5. OPC/OC cultures were supplemented with CSF-1 (30 ng/mL) and RANKL (20 ng/mL) to stimulate OPC/OC development and maturation. Statistical differences were determined using the Kruskal-Wallis test with Dunn test by comparing to the control group (0 μM). Differences were termed statistically significant with values of $P \leq 0.05$ (*), $P \leq 0.01$ (**), and $P \leq 0.001$ (***). Data presented as mean ± standard deviation with 4–6 biological replicates (n = 4–6) in technical replicates of 6. GBCA, gadolinium-based contrast agent; OPC, osteoclast progenitor cell; OC, osteoclast; TRAP, tartrate-resistant acid phosphatase; CSF-1, colony-stimulating factor 1; RANKL, receptor activator of nuclear factor-κB ligand.

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is possible that the negative effects of DTPA are restricted to in vitro systems, as it may form complexes with zinc ions by transmetalation[17] and thus deplete the culture medium from zinc ions, a co-factor essential for cell and enzyme functions.[41] Our data suggest that the different reactions of OB in vitro to the exposure to empty chelators are caused by the structural properties of the complexing molecules, as the linear agent DTPA is more prone to bind metal ions from the culture medium than the macrocyclic DOTA.[17,25]

In OPC cultures supplemented with Gd^{3+}, cell viability was increased, whereas cell differentiation was inhibited or delayed. Similar results were obtained in cultures supplemented with GBCA (Gd-DTPA > - Gd-DOTA > Gd-DTPA-BMA). This outcome does not correlate with the thermodynamic stability of the contrast agents. Hence, the effects of GBCA may be partially attributable to the chelating molecules, as Gd^{3+} alone did not affect OPC/OC cultures to a similar extent. As Ca^{2+}-signaling is a crucial regulator in both OB and OC function,[42,43] in OPC/OC cultures, Gd^{3+} may act on the extracellular Ca^{2+}-sensing receptor, a G-protein–coupled receptor essential for the transmission of Ca^{2+} signals, without initiating downstream signaling that promotes cell maturation or apoptosis.[44] This could explain the prolonged lifespan accompanied by attenuation of cell differentiation observed in cultures treated with Gd^{3+} and GBCA.

It is well-documented that Gd is deposited in bone[20,22,24] and might form a composite of Gd and hydroxyapatite. To assess the effects of mineral-bound Gd on bone turnover, Gd-enriched amorphous CaP layers were generated. The high affinity of Gd to inorganic phosphate led to almost complete incorporation during the precipitation process of the amorphous CaP. Gadolinium incorporation through aqueous precipitation was already applied during the manufacturing of β-tricalcium phosphate [β-$Ca_3(PO_4)_2$], leading to alterations in the crystal structure of the material.[45] The capacity of mature OC to dissolve the CaP mineral was significantly reduced upon incorporation of 4.3% Gd/Ca wt/wt. Whether the solubility of the Gd-CaP mixture differs from that of amorphous CaP or whether the internalized Gd leads to reduced OC activity remains to be elucidated. Previous data suggested either an inhibition of the resorptive activity of OC cultures in the presence of Gd^{3+} (200 μM)[46] or no effects caused by Gd^{3+} (10 μM).[47] Both results corroborate with our study, showing that the effects of Gd on bone cells are dose-dependent, with 10 μM exerting only marginal effects.

In the present study, the effects of gadolinium-based contrast agents, empty chelator molecules, and free Gd^{3+} on bone cells were investigated. Differentiation of the OB and OC lineages was inhibited in a dose-dependent manner by free Gd^{3+} and GBCA. The documented effects in OB cultures are primarily the result of dechelated Gd^{3+}, with the most significant impact observed with nonionic and ionic linear GBCA. In OPC/OC cultures, the negative effects seem to be associated with free Gd^{3+} and, to some degree, empty chelator molecules, as evidenced by the stronger inhibition of OPC/OC development by Gd-DTPA and Gd-DOTA compared with Gd-DTPA-BMA. Furthermore, Gd^{3+} incorporation into amorphous CaP reduced the resorbability of the mineral layer by mature OC. Hence, Gd stored in the bone, which is released during tissue turnover, can cause an imbalance between bone formation and resorption, impairing the physiological regulation of bone turnover and calcium homeostasis. Gadolinium release may lead to constant systemic exposure when distributed via circulation. Additional investigations, both ex vivo and in vivo,

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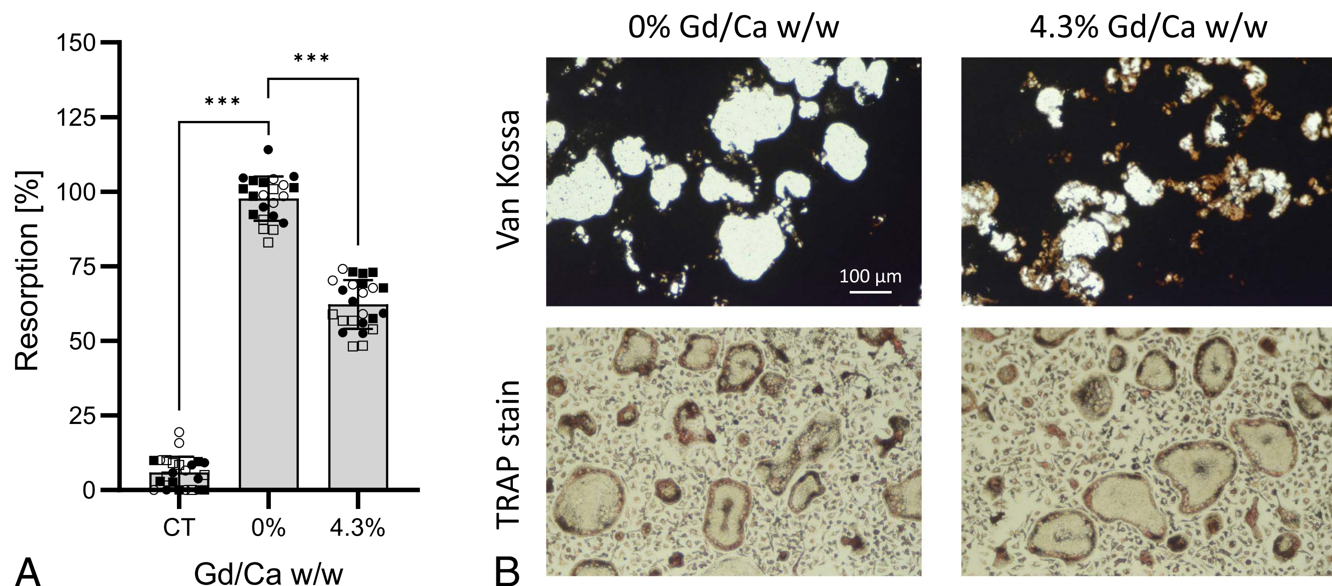

FIGURE 6. Effect of Gd incorporation into CaP on the capacity of OC to dissolve the mineral. Effect of Gd incorporation into amorphous CaP layers spiked with ^{45}Ca on the dissolution capacity of mature of OC (A). CaP layers with 4.3% Gd/Ca wt/wt were generated. Resorption by OC is relative to the dissolution of CaP without Gd incorporation and displayed in %. The resorption by OC is calculated by the release of ^{45}Ca into the cell culture medium and normalized to the TRAP activity of the respective cell culture. Von Kossa staining of remaining CaP and TRAP stain of multinucleated OC on CaP layers (B). Von Kossa silver staining visualizes the remaining CaP in black and resorption pits appear white during light microscopy. In addition, OC cultures were supplemented with CSF-1 (30 ng/mL) and RANKL (20 ng/mL) to stimulate the resorptive activity of the cells. CT was added in the negative control group to inhibit the resorptive activity of OC. Statistical differences were determined using the Kruskal-Wallis test with Dunn test by comparing to the control group (0% Gd/Ca wt/wt). Differences were termed statistically significant with values of $P \leq 0.001$ (***). Data are presented as the mean ± standard deviation in biological replicates of 4 (n = 4) in technical replicates of 6. Biological replicates are depicted with different symbols. OC, osteoclast; CaP, calcium phosphate; TRAP, tartrate-resistant acid phosphatase; CSF-1, colony-stimulating Factor 1; RANKL, receptor activator of nuclear factor-κB ligand; CT, calcitonin.

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are necessary to further elucidate the risks associated with accumulation of Gd and GBCA in the bone and other organ systems.[40]

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