Gallium enhances reconstructive properties of a calcium phosphate bone biomaterial

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Abstract

Calcium phosphate (CaP)‐based biomaterials are commonly used in bone reconstructive surgery to replace the damaged tissue, and can also serve as vectors for local drug delivery. Due to its inhibitory action on osteoclasts, the semi‐metallic element gallium (Ga) is used for the systemic treatment of disorders associated with accelerated bone resorption. As it was demonstrated that Ga could be incorporated in the structure of CaP biomaterials, we investigated the biological properties of Ga‐loaded CaP biomaterials. Culturing bone cells on Ga‐CaP, we observed a decrease in osteoclast number and a downregulation of late osteoclastic markers expression, while Ga‐CaP upregulated the expression of osteoblastic marker genes involved in the maturation of bone matrix. We next investigated in vivo bone reconstructive properties of different Ga‐loaded biomaterials using a murine bone defect healing model. All implanted biomaterials showed a good osseointegration into the surrounding host tissue, accompanied by a successful bone ingrowth and bone marrow reconstruction, as evidenced by histological analysis. Moreover, quantitative micro‐computed tomography analysis of implants revealed that Ga enhanced total defect filling. Lastly, we took advantage for the first time of a particular mode of non‐linear microscopy (second harmonic generation) to quantify in vivo bone tissue reconstruction within a CaP bone substitute. By doing so, we showed that Ga exerted a positive impact on mature organized collagen synthesis. As a whole, our data support the hypothesis that Ga represents an attractive additive to CaP biomaterials for bone reconstructive surgery. Copyright © 2017 John Wiley & Sons, Ltd.

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Introduction

In the field of bone tissue engineering, major efforts are currently focused on the development of novel biomaterials usable for bone reconstruction. Calcium phosphate (CaP) biomaterials are considered the best alternative to bone grafting because of their biocompatibility and chemical properties suitable for bone remodelling (LeGeros, 1988; Libicher et al., 2006; Ooms et al., 2003a, b; Rush, 2005; Tomita et al., 2003; Welch et al., 2003). Among them, CaP cements (CPCs), based on studies performed originally by LeGeros in 1982 (LeGeros, 1982) and Brown and Chow in 1986 (Brown and Chow, 1986), have considerably revolutionized the world of bone tissue engineering, due to their mouldability, injectability, rapid integration into the bone structure and progressive resorption rate. Different injectable biodegradable CPCs have been developed and commercialized for dental and orthopaedic indications in both EU and US markets since 2009.

After implantation into the bone defect, CPCs are able to self‐set (Chen et al., 2003) and harden without any significant exothermic reaction that could be prejudicial to the bone tissue and/or loaded drug. Thus, CPC combined devices loaded with active molecules represent good candidates for sustained local drug delivery (Ginebra et al., 2012).

In the early 1970s, it was demonstrated that the semi‐metallic element gallium (Ga) had several therapeutic effects [for a review see Chitambar (2010)], including: (i) the inhibition of neoplastic proliferation; (ii) the treatment of some pathogens such as species of Mycobacterium (Bernstein, 1998); (iii) the decrease of accelerated bone mineral resorption, which occurs during osteolytic bone diseases, and the subsequent lowering of elevated plasma calcium levels associated with these pathologies. In this context, Ga has been used to treat hypercalcaemia resulting from malignant tumours such as parathyroid carcinoma, and Paget’s disease of bone (Warrell et al., 1984, 1990, 1988). This anti‐hypercalcaemic effect of Ga results from
Gallium stimulates bone reconstruction

...an inhibition of bone resorption (Bockman, 2003; Warrell et al., 1984). Indeed, due to its chemical characteristics, Ga presents an affinity for biological apatite, which explains the presence of Ga deposits in bone tissue, and preferentially at sites of rapid bone remodelling, such as active metaphyseal growth plate and healing fractures (Bockman et al., 1986, 1990; Korbas et al., 2004).

Few studies have explored the effect of Ga on bone cells. Hall and Chambers (1990) demonstrated that ionic Ga inhibited bone resorption in a dose-dependent manner and reduced the osteoclastic resorption activity without inducing modifications to the morphology or number of osteoclasts. In contrast to these findings, Blair et al. (1992) showed a cytotoxic effect of Ga on osteoclasts. Considering this discrepancy, we previously investigated the biological effect of Ga on bone cells and reported that Ga, administered in cell culture medium, inhibited both the differentiation and the resorbing activity of osteoclasts in a dose-dependent manner (0–100 μM) without affecting the viability or proliferation of osteoblasts (Verron et al., 2010, 2012b).

Furthermore, studies by Ma et al. (2010a, b) on ovariectomized osteopenic rats showed positive effects of orally administrated Ga on trabecular bone volume, trabecular and cortical thickness, bone mineral content of the vertebrae and femoral neck strength. Similarly, Stern et al. (1994) demonstrated that Ga administration stimulates osteoblast activity in ovariectomy-induced osteopenia associated with a low calcium diet. Nevertheless, this stimulation was not sufficient to prevent osteopenia bone loss in rats. Another study using organic yeast-derived Ga showed that Ga can promote callus bone formation in a fracture healing model in ovariectomized rats (Pei and Fu, 2011). A study in normal rats where Ga was injected intraperitoneally showed lower calcium and vitamin D serum levels, and inhibition of bone resorption, but also bone formation (Wakeley et al., 1992). In addition, Jenis et al. (1993) found that a subcutaneous 3 week Ga treatment decreased calcium serum levels without any significant effect on bone mineral density in normal rats. The discrepancy in Ga effects on bone in the abovementioned studies could be due to the differences in Ga treatment duration, doses and administration routes, as well as to the fact that some studies used osteoporotic models, whereas others were conducted in rats with normal bone turnover.

Several clinical trials confirmed that Ga was effective against cancer-induced hypercalcemia resulting from an excessive bone resorption (Collery et al., 2002). Using gallium nitrate alone or combined with other chemotherapeutic agents, significant positive outcomes have been obtained, with patients experiencing recurrence or for patients who responded poorly to conventional chemotherapy (Chitambar, 2012). For example, a small clinical trial involving myeloma patients receiving chemotherapy showed that Ga could attenuate the rate of bone loss (Niesvizky, 2003). As a whole, these data strongly support the therapeutic potential of Ga compounds in the treatment of bone turnover disorders (Verron et al., 2012a).

However, some studies have reported that the systemic administration (oral or parenteral) of Ga compounds results in elevated concentration of Ga in kidneys and liver, possibly generating toxicity issues together with a poor delivery of Ga to the aimed tissue (Verron et al., 2012a). These important drawbacks related to systemic administration could be circumvented by a local Ga delivery. Mellier et al. (2011, 2015) have defined suitable protocols for inserting Ga ions into CaP matrices, while generally preserving their textural, mechanical or setting properties. More recently, the same authors assessed the performance of two different types of Ga-loaded CPCs in a rabbit femoral defect model (Mellier et al., 2015) with no significant difference observed 4 weeks after implantation between control and the Ga-containing formulations. The authors underlined that the amount of resorbed biomaterials was quite low, and as a consequence the Ga release, suggesting longer-term implantations in different animal models in order to investigate whether the presence of Ga might improve bone regeneration. With this scope and in the light of our previous findings on the Ga effect on bone cells, when administered in cell culture medium, in the present work we wanted to evaluate the performance of different CaP bone biomaterials as vectors for sustained Ga delivery. For that purpose, we used Ga-loaded CaP pellets as a substrate for bone cell culture and Ga-loaded CaP microparticles for in vivo bone tissue reconstruction assays.

Materials and methods
Biomaterial synthesis and preparation

Graftys SARL (Aix-en-Provence, France) provided all the biomaterials used in this study. A very detailed account of biomaterial synthesis and their physicochemical characterization is described in Mellier et al. (2011, 2015). A short overview of the synthesis and processing of the biomaterials is presented in Figure 1. Briefly, the solid phase of control CPC consisted of α-tricalcium phosphate (α-TCP, Ca₃(PO₄)₂, 78 wt.%), dicalcium phosphate anhydrous (DCPA, CaHPO₄, 10 wt.%), calcium-deficient apatite (CDA, Ca₁₀₋ₓ[Ca⁺ₐₐ](HPO₄)₃₋ₓ(OH)ₓ₋₄, 10 wt.%) and polymer hydroxypropyl methyl cellulose (HPMC, 2 wt.%). For Ga-loaded CPC (CPC/Ga), the CDA part of the cement powder was loaded with Ga ions by surface adsorption, as previously described in Mellier et al. (2011, 2015). Briefly, CPC/Ga was synthesized by co-precipitation from a mixture of gallium nitrate hydrate, calcium nitrate tetrahydrate and diammonium hydrogen phosphate with the (Ca + Ga)/P molar ratio at 1.51 and the Ca/Ga molar ratio in the 0–0.08 range. At the end of synthesis, the white precipitate was dried at 70°C for 24 h. The amount of Ga incorporated in the isolated solid phase was 3 wt.%, as determined by atomic absorption spectroscopy and X-ray
diffraction (Mellier et al., 2011). This CDA/Ga comprised 10 wt.% of the solid phase of CPC.

For in vitro experiments, the solid powder phase of CPC or CPC/Ga was mixed for 2 min with its liquid phase, consisting of a 0.5 wt.% aqueous solution of Na$_2$HPO$_4$ (Merck, Darmstadt Germany) in a liquid/solid ratio of 0.45 mlg$^{-1}$, according to manufacturer’s instructions. The obtained paste was placed in silicon moulds and set at 37°C in physiological saline solution for 72 h. For details on X-ray diffraction patterns and solid-state nuclear magnetic resonance analysis of the biomaterials, please refer to Mellier et al. (2011, 2015). Then, solidified pellets with a surface of approximately 0.5 cm$^2$ were polished and cleaned from dust three times for 15 s in an ultrasound bath. Pellets were further steam sterilized at 121°C for 20 min and incubated for 48 h prior to cell seeding in α-MEM medium supplemented with 10% Hyclone FCS, 100 U/ml penicillin, 100 U/ml streptomycin and 2% ultraglutamine 1. For experiments, human primary osteoblasts (#CC-2538, Lonza) were expanded to P7 at 6000 cells/cm$^2$. Human primary monocytes were isolated from whole blood samples of healthy individuals (provided by Etablissement Français du Sang La Plaine St Denis, France) using Uni-SepMaxi tubes (NOVAMED Ltd, Jerusalem Israel) and Easy Sep Human Monocyte Enrichment Kit (STEMCELLS Technologies, Vancouver Canada) according to manufacturers’ instructions. After seeding freshly isolated monocytes, the differentiation towards osteoclasts was triggered by adding 66 ng/ml receptor activator of nuclear factor kappa B ligand (RANKL) (Peprotech, Neuilly sur Seine France) and 33 ng/ml macrophage-colony stimulating factor (M-CSF) (Peprotech) simultaneously to the culture medium. After each medium renewal, RANKL and M-CSF were added at the same concentrations as after cell seeding. For co-culture experiments osteoblasts were seeded at 100 000 cells/pellet and 4 hours later (after osteoblastic adhesion), monocytes were added at 200 000 cells/pellet. In co-culture experiments no exogenous ligands were added to the culture medium. Both for monoculture and for co-culture, the culture medium was renewed every third day.

Cell culture

Both cell types used in this study, primary human osteoblasts and monocytes, were cultured in α-MEM medium supplemented with 10% Hyclone FCS, 100 U/ml penicillin, 100 U/ml streptomycin and 2% ultraglutamine 1. For experiments, human primary osteoblasts (#CC-2538, Lonza) were expanded to P7 at 6000 cells/cm$^2$. Human primary monocytes were isolated from whole blood samples of healthy individuals (provided by Etablissement Français du Sang La Plaine St Denis, France) using Uni-SepMaxi tubes (NOVAMED Ltd, Jerusalem Israel) and Easy Sep Human Monocyte Enrichment Kit (STEMCELLS Technologies, Vancouver Canada) according to manufacturers’ instructions. After seeding freshly isolated monocytes, the differentiation towards osteoclasts was triggered by adding 66 ng/ml receptor activator of nuclear factor kappa B ligand (RANKL) (Peprotech, Neuilly sur Seine France) and 33 ng/ml macrophage-colony stimulating factor (M-CSF) (Peprotech) simultaneously to the culture medium. After each medium renewal, RANKL and M-CSF were added at the same concentrations as after cell seeding. For co-culture experiments osteoblasts were seeded at 100 000 cells/pellet and 4 hours later (after osteoblastic adhesion), monocytes were added at 200 000 cells/pellet. In co-culture experiments no exogenous ligands were added to the culture medium. Both for monoculture and for co-culture, the culture medium was renewed every third day.
Cell viability assays

Human primary osteoblasts at P2 were seeded at 50 000 cells/pellet and freshly isolated human primary monocytes at 90 000 cells/pellet for cell viability assessment in monoculture. For co-culture conditions, 100 000 osteoblasts and 200 000 monocytes/pellet were seeded. At days 3, 6 or 14 of culture, cells were rinsed twice in phosphate-buffered saline (PBS; Lonza) and incubated in 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) for 1 h in a cell incubator. MTT was aspirated delicately and 0.3 ml lysis buffer (sodium dodecyl sulphate 10%, HCl 0.01 N) was added/well and left overnight at room temperature, in the dark. The next morning, supernatants were centrifuged 2 min at 20 000 g and absorbance was measured in 100 μl at 562 nm. In parallel experiments, using increasing amounts of cells cultured on plastic or pellets, we verified the relationship between cell number and MTT assay result (data not shown).

Tartrate-resistant acid phosphatase (ACP5) staining

Human primary monocytes were seeded alone at 200 000 cells/pellet or co-cultured with 100 000 human primary osteoblasts, without the addition of osteoclast differentiation ligands. For negative staining control, 100 000 osteoblasts/pellet were seeded alone. After 7 or 14 days, cells were rinsed two times in PBS and fixed in citrate/acetic solution for 30 s. ACP5+ cells were revealed using the acid phosphatase, leukocyte kit following the manufacturer’s instructions (# 387A; Sigma-Aldrich).

Gene expression analysis

For monoculture experiments, 100 000 human primary osteoblasts or 200 000 freshly isolated human primary monocytes were seeded on plastic or biomaterial pellets. For co-culture conditions, 100 000 osteoblasts and 200 000 monocytes/pellet were seeded. At days 2, 5, 7 or 14, cells were rinsed twice in PBS and incubated in 0.25 % trypsin/ethylene diamine tetra acetic acid (EDTA; Lonza) at 37°C for 5 min. Total RNA was extracted using the NucleoSpin RNA XS kit (Macherey-Nagel, Duren Germany) following manufacturer’s instructions and RNA concentration was measured on Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham USA). 150 ng RNA was used as a template for cDNA synthesis using random primers and SuperScript II reverse transcriptase (Invitrogen, Waltham USA) in Flexycycler (Analytik Jena, Jena Germany). Total cDNAs were diluted 10 times and 5 μl template was used for quantitative real-time polymerase chain reaction (PCR) assay (StepOne Plus, Life Technologies, Waltham USA). Reactions were performed in a 20 μl final volume with quantitative PCR mastermix GoTaq (Promega, Madison USA). Amplification conditions were as follows: 2 min at 50°C, 10 min at 95°C, 40 cycles (15 s at 95°C, 1 min at 60°C). The expression of genes of interest was normalized to ribosomal protein large P0 (RPLP0) gene expression, quantified by the comparative 2^Ct method and expressed as a fold change compared with control conditions without Ga. The primers used in quantitative real-time PCR are listed in Table 1.

Field-emission scanning electron microscopy analysis

Human primary osteoblasts and human primary monocytes were mono- or co-cultured as described above. Cells seeded on biomaterial pellets were fixed overnight at 4°C in a buffered glutaraldehyde solution, rinsed, dehydrated in a graded ethanol series, immersed in hexamethyldisilazane (Sigma-Aldrich) for 5 min and dried at room temperature. The samples were then mounted on aluminium stubs and sputter coated with gold-palladium (Cressington, 308R, Watford UK). An examination was performed using a field-emission scanning electron microscopy (FESEM JEOL 6700F, Tokyo, Japan).

Orthotopic implantation of biomaterial microparticles in rat

All animal procedures obtained the approval of the local animal health care committee (CIEPAL NCE/2012-69) and were conducted in accordance with European Community guidelines for the care and use of laboratory animals (Directive 2010/63/UE). Bilateral femoral implantations of biomaterial microparticles were performed on 16 male Lewis rats weighing approximately 300 g (Janvier Labs, Le Genest-Saint-Isle France), in aseptic conditions under general anaesthesia induced by

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ACPS, acid phosphatase 5 tartrate resistant; ALPL, alkaline phosphatase; BGLAP, bone gamma-carboxyglutamate (Gla) protein (osteocalcin); COL1A1, collagen type I alpha 1; CTSK, cationic trypsin; FBJ, F-box; IBSP, integrin-binding sialoprotein; ITGB3, integrin, beta 3; JDP2, jun dimerization protein 2; MCSF, macrophage colony-stimulating factor 1; MMP9, matrix metalloproteinase 9; NFATC1, nuclear factor of activated T-cells, cytoplasmic 1; ON, osteonectin; OPG, osteoprotegerin; OPN, osteopontin; POSTN, periostin osteoblast specific factor; RANKL, receptor activator of nuclear factor kappa-B ligand; RPLP0, ribosomal protein large P0; RN2X2, nuc-related transcription factor 2.
inhalation of isoflurane 5% and maintained by intramuscular injections of xylazine 10 mg/kg and ketamine 50 mg/kg. The implants were prepared as previously described (Balaguer et al., 2010; Boukhechba et al., 2011). Briefly, in 1 ml syringes (Therumo Europe N.V., Leuven Belgium) 60 mg CPC, CPC/Ga, CDA or CDA/Ga 80–200 μm microparticles (previously heat sterilized at 180°C for 3 h) were extemporaneously mixed with 100 μl previously prepared rat platelet-poor plasma. The coagulation of plasma was triggered by adding 10 μl 2% CaCl₂, 2 H2O solution (Merck, Germany) and 1 U thrombin (Sigma-Aldrich). After at least 15 min, the tops of the syringes were cut off and coagulated microparticles were implanted with a spatula in a 3 × 2.7 mm cylindrical defect drilled at the distal epiphysio-metaphysal junction in rat femora. In the first set of experiments, six animals were implanted with CPC or CPC/Ga contralateral; in the second set, 10 animals were implanted with CDA or CDA/Ga contralateral. After 2 months, animals were sacrificed by inhalation of CO₂ and the bone specimens were retrieved immediately and stored in 70% ethanol.

Three-dimensional microtomography analysis

Physiological saline-immersed rat femora were scanned with SkyScan 1172 microtomograph (Bruker, Billerica USA) equipped with 12-bit digital CCD camera. Samples were placed with the axis of the femur perpendicular to the plane of scanning and cross-section images were acquired under 90 kV voltage and 88 μA current with a 1 mm aluminium filter. The rotation step was set at 0.7° and the exposure time per step was 776 ms. Three-dimensional reconstruction of 6 μm resolution cross-section images was carried out in NRecon software (Bruker) using a modified Feldkamp cone-beam algorithm, as provided by the manufacturer. The parameters were set as follows: smoothing at 3, ring artefact correction at 10, beam hardening correction at 50%, rotation angle 180° and image conversion values in ranges 0.003–0.03 ranges. To quantify bone regeneration and biomaterial degradation in the defects, the region of interest was set to the whole implant area excluding the cortical part in CTAn analysis software (Bruker).

Histological preparation and analysis

CPC and CPC/Ga explants were fixed for 24 h in neutral formalin solution (pH 7.2) and then dehydrated in baths with ethanol concentration increasing from 70% up to 100% for 3 days/bath. Bone specimens were then infiltrated and embedded in methyl methacrylate, as previously described (Verron et al., 2010). Undecalcified serial 5 μm sections were cut using a hard tissue microtome (Supercut 2050, Reichert-Jung, Depew, USA) equipped with a D profile tungsten carbide knife. These sections were stained with haematoxylin/eosin (HE) and assessed to visualize mature bone, osteoid tissue, bone marrow and residues of biomaterial microparticles. CDA and CDA/Ga explants were fixed for 24 h in neutral formalin solution (pH 7.2) and then decalcified in 10% EDTA (pH 8) for 4 weeks. These samples were embedded in paraffin and 5 μm thick sections were prepared and stained with HES to visualize the relevant bone structures and biomaterial microparticles. All histological sections were imaged by Nikon Eclipse 80i (Nikon, Tokyo, Japan) for qualitative observations.

Second harmonic generation (SHG) preparation and analysis

SHG microscopy relies on a highly specific optical phenomenon produced in a very limited number of biological structures (type I collagen fibres, myosin II fibres and tubulin) (Vielreicher et al., 2013). Imaging type I collagen by SHG microscopy requires excitation laser wavelengths from 720 to 960 nm, while the generated SHG signal is exactly half of the excitation wavelength. For bone tissue, SHG microscopy’s ability to detect predominantly organized collagen fibres has been experimentally validated by several comparative studies using techniques such as immunofluorescence staining light microscopy, polarized light microscopy and scanning electron microscopy (Ambekar et al., 2012). As SHG has intrinsic specificity for collagen fibres, this results in high contrast images, with no additional staining needed. Thus, tissue sections prepared (as described above) for histological study were also used for SHG imaging (Zeiss LSM 710 microscope, Oberkochen Germany). The Mai Tai laser excitation wavelength was set to 880 nm and the SHG signal was detected in transmission and reflection mode. For CPC and CPC/Ga, 5 μm sections were qualitatively analysed; for CDA and CDA/Ga explants, additional 100 μm sections were cut to carry out quantification on a larger portion of the samples. Quantitative comparison of collagen signal between Ga and control samples was performed on the whole implant area using an in-house quantification tool developed in Matlab programming language by S. Schaub from iBV PRISM platform (Platform of Resources in Imaging and Scientific Microscopy).

Statistical analysis

In vitro results are expressed as a mean ± standard deviation of three independent experiments each performed in triplicate. Differences between Ga and the control group were evaluated by a bidirectional Mann and Whitney U test with the risk threshold set at 5%. For microtomography experiments, significant differences in total defect filling between Ga and control groups were determined by bidirectional Kruskal-Wallis one-way analysis of variance with the risk threshold set at 5%. For SHG quantitative analysis, a bidirectional Mann and Whitney U test with the risk threshold set at 5% was
used to assess significant differences between Ga and control biomaterials.

Results

Human primary bone cells culture on apatitic matrices loaded with Ga

Human primary monocytes were seeded on CPC or CPC/Ga pellets and cultured for 3 or 6 days before an MTT assay was performed. In parallel experiments (data not shown), we verified that MTT assay results correlated with the number of viable cells seeded on apatitic matrices. As shown in Figure 2A, and compared with the CPC control condition, seeding monocytes on CPC/Ga resulted in slightly decreased cell viability/number for both time points. We also performed a cell structure examination using scanning electron microscopy analysis. As depicted in Figure 2B, we observed monocytes on CPC and CPC/Ga (white arrowheads), as well as cellular extensions interacting with the cement surface. In the presence of the osteoclastogenic agent RANKL, few cells exhibiting osteoclast-like morphology were also observed. However, these events remained rare for both cements. Lastly, we performed quantitative real-time PCR experiments to quantify the expression of early and late osteoclastogenesis marker genes (gene symbols are

![Graph and figure captions]

Figure 2. Cellular proliferation, morphology and differentiation of human primary monocytes on calcium phosphate cement (CPC) or gallium-loaded CPC (CPC/Ga) pellets. (A) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. 90 000 monocytes were cultured for 3 or 6 days. Data represent the mean ± standard deviation of three independent experiments, with each including five MTT measures. (B) Field-emission scanning electron microscopy analysis of pellets seeded with monocytes and cultured for 5 days in the presence of hM-CSF (33 ng/ml; upper panels) or in the presence of hM-CSF/hRANKL (33 ng/ml and 66 ng/ml, respectively; lower panels). The white arrowheads indicate cells (magnification 2500×, scale bars: 10 μm). (C) Real-time polymerase chain reaction (PCR) quantification of osteoclastogenesis markers. Monocytes were cultured for 2 days (NFATC1, JDP2) or 5 days (ACP5, MMP9, CTSK), in the presence of 33 ng/ml hM-CSF (control) or 33 ng/ml hM-CSF and 66 ng/ml hRANKL (RANKL). Data represent the mean ± standard deviation of three independent experiments, each performed in triplicate. Data were normalized with respect to gene expression on plastic/control or on CPC/control for cell culture on cement pellets. a: statistically significant compared with plastic/control or CPC/control (p < 0.05, bi-directional analysis).
defined in the Materials and methods section). As shown in Figure 1C, RANKL induced the differentiation of monocytes seeded on plastic, as attested by the expression of early (NFATC1, JDP2) and late (ACP5, MMP9, CTSK) marker genes. By contrast, RANKL failed to induce any osteoclastic differentiation of monocytes seeded either on CPC or CPC/Ga. We also observed that, whatever the condition (control or RANKL), the expression of the transcription factors JDP2 and FBJ was diminished on CPC/Ga, whereas the late marker genes MMP9 and CTSK were induced in the presence of Ga. This first set of experiments indicated that human primary monocytes did not efficiently differentiate into osteoclasts when seeded alone on CPC or CPC/Ga.

We next seeded human primary osteoblasts on CPC or CPC/Ga and quantified cell viability/number. As shown in Figure 3A, after 3 days of culture, the presence of Ga induced a 40% inhibition of cell viability/number. At day 7, cellular proliferation was observed on both cements, whereas the inhibition observed in the presence of Ga tended to decline (30% instead of 40%). Cell structure was also examined using scanning electron microscopy analysis. As shown in Figure 3B, osteoblastic morphology remained unchanged when cells were cultured on CPC/Ga compared with control CPC. Lastly, we used quantitative real-time PCR experiments to quantify the expression of osteoblast marker genes (gene symbols are defined in the Materials and methods section) and three kinds of situation were observed (Figure 3C). Firstly, RUNX2, ALPL and OPN were expressed at similar levels at both times on the control cement. Considering the CPC/Ga, the expression of these three genes was inhibited at day 3, and this inhibition tended to decrease at day 7. Second, BGLAP expression increased between day 3 and day 7, and no difference was observed comparing CPC and CPC/Ga. Third, COL1A1, IBSP, ON and POSTN expression was identical on CPC or CPC/Ga at day 3. At day 7, the expression level of the four genes decreased on both cements (except for POSTN gene on CPC/Ga) but remained at higher levels on CPC/Ga compared with control CPC. Taken together, these data indicated that following 7 days of primary osteoblast culture on

![Figure 3. Cellular proliferation, morphology and differentiation of human primary osteoblasts on calcium phosphate cement (CPC) or gallium-loaded CPC (CPC/Ga) pellets. (A) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay: 50 000 osteoblasts were cultured for 3 or 6 days. Data represent the mean ± standard deviation of three independent experiments, with each including five MTT measures. (B) Field-emission scanning electron microscopy analysis of osteoblasts cultured for 5 days on pellets. The white arrowheads indicate cells (magnification 500×, scale bars: 10 μm). (C) Real-time polymerase chain reaction (PCR) quantification of osteoblastic markers after 3 or 7 days of cell culture. Data represent the mean ± standard deviation of three independent experiments, each performed in triplicate. Data were normalized with respect to gene expression on CPC at day 3. a: statistically significant compared with CPC/day 3 (p < 0.05, bi-directional analysis).]
cements, the expression level slightly diminished on CPC/Ga for RUNX2 and OPN, was identical for ALPL and BGLAP and was enhanced on CPC/Ga for COL1A1, ISBP, ON and POSTN.

To document monocyte/osteoblast interactions, human primary osteoblasts and human primary monocytes were then co-cultured up to 14 days on both cements, in the absence of any additional effectors such as M-CSF and RANKL. As depicted in Figure 4A, cell viability/number decreased between day 3 and day 7, and in a lesser extent between day 7 and day 14. Except for day 3, no difference was observed between the two cements. Regarding cellular

![Graph showing MTT assay results](image)

### Figure 4. Cellular proliferation, morphology and differentiation of human primary monocytes co-cultured with human primary osteoblasts on calcium phosphate cement (CPC) or gallium-loaded CPC (CPC/Ga) pellets. (A) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay: 100 000 osteoblasts and 200 000 monocytes were co-cultured for 3, 6 or 14 days. Data represent the mean ± standard deviation of three independent experiments, with each including five MTT measures. (B) Field-emission scanning electron microscopy analysis of osteoblasts and monocytes co-cultured for 14 days on pellets. The white and red arrowheads indicate osteoblasts and osteoclasts, respectively (magnification 500×, scale bars: 10 μm). (C) Histochemical staining of differentiated monocytes positive (red arrowheads) for ACP5 activity. 100 000 osteoblasts were cultured alone or in the presence of 200 000 monocytes for 14 days (scale bars: 100 μm). (D) Real-time polymerase chain reaction (PCR) quantification of osteoclastic markers after 6 or 14 days of osteoblast culture or osteoblasts/monocytes co-culture. Data represent the mean ± standard deviation of three independent experiments, each performed in triplicate.

For each condition (osteoblasts or co-culture) data were normalized with respect to gene expression on CPC. a: statistically significant compared with CPC (p < 0.05, bi-directional analysis).
morphology (Figure 4B), we observed osteoblasts on both surfaces (white arrowheads), as well as monocytes differentiating into osteoclasts (red arrowheads). We next performed a histochemical demonstration of ACP5 (also known as TRAP) activity. As shown in Figure 4C (left panels), osteoblasts were stained in yellow/brown (white arrowheads) and no difference was observed on CPC/Ga compared with CPC. Regarding osteoblasts/monocyte co-culture, a purple staining was observed within osteoclasts (right panels, red arrowheads) on both cements, but the number of differentiated cells was decreased on CPC/Ga compared with CPC. To further document this inhibition at the molecular level, we eventually performed quantitative real-time PCR experiments (gene symbols are defined in the Materials and methods section) using RNAs prepared from osteoblasts alone or from osteoblasts co-cultured with monocytes (Figure 4D). Concerning the transcription factors involved in the early steps of osteoclastogenesis, we observed that NFATC1, JDP2 and FBJ were expressed in both conditions (osteoblasts alone vs. co-culture), with no significant differences between CPC and CPC/Ga. The same situation was observed for the late marker genes of osteoclastic differentiation, ITGB3 and CTSK. By contrast, the late marker genes ACP5 and MMP9 were detected only in co-culture conditions and not in osteoblast alone conditions, suggesting an expression restricted to monocytes differentiated into osteoclasts. In this co-culture situation, ACP5 and MMP9 expression was strongly inhibited on CPC/Ga compared with CPC. Lastly, we also tested the expression of RANKL, MCSF and OPG, the triad of osteoblastic genes regulating osteoclastic differentiation. Considering the co-culture condition, we showed that no difference could be observed between CPC and CPC/Ga. In conclusion, we observed that ACP5 activity histochemical staining revealed an inhibition of osteoclastic differentiation on CPC/Ga compared with CPC. Moreover, this inhibition could also be quantified by quantitative real-time PCR experiments measuring the expression of marker genes restricted to differentiated osteoclasts, i.e. ACP5 and MMP9.

Orthotopic in vivo implantation of apatitic matrices loaded with Ga

We next performed in vivo implantation experiments to determine Ga impact on the reconstructive properties of the CPC under study. As illustrated in Figure 5A, cements were first processed into 80–200 μm diameter micro-particles and then implanted within cylinder defects generated in the distal part of rat femurs. After 2 months, implants were retrieved and processed to perform HE staining and SHG analysis on tissue sections, as well as micro-computed tomography quantification of the whole implants. Both HE staining (Figure 5B) and SHG (Figure 5C) methods demonstrated for CPC and CPC/Ga a successful bone reconstruction including newly formed tissue up to the central part of implants and a complete cortical bone restoration, without any inflammatory response or fibrous encapsulation. Based on image

Figure 5. Femoral implantation in rat of calcium phosphate cement (CPC) or gallium-loaded CPC (CPC/Ga) 2 months after implantation. (A) Qualitative three-dimensional micro-computed tomography; (B) HE histological staining; (C) second harmonic generation analysis; (D) quantitative three-dimensional micro-computed tomography analysis; (E) total defect filling quantification: results are expressed as total defect filling rate (CPC, n = 6; CPC/Ga, n = 6)
acquisitions illustrated in Figure 5D, micro-computed tomography was used to quantify total defect filling. The results presented in Figure 5E did not show any significant impact of Ga on bone reconstruction.

With a view to increase Ga content in biomaterial used for bone defects filling, we shifted from CPC to CDA loaded or not with Ga. Indeed, as detailed in the Materials and methods section, Ga was brought to CPC through the CDA part (10%) of the cement solid phase formulation. Thus, using pure CDA allowed increasing Ga content by a factor of 10. As illustrated by HE staining and SHG analysis (Figure 6A, B), and similarly to CPCs, CDA and CDA/Ga implantations led after 2 months to good osseointegration into the surrounding host tissue, accompanied by a successful bone ingrowth and bone marrow reconstruction on the border of the implants. Taking advantage of the unique ability of SHG to specifically detect fibrillar collagen in bone tissue and to carry out quantification on larger implant volumes, we performed 100 μm sections (compared with classical 5 μm histological sections). As shown in Figure 6B (right panel), we measured a 74% increased SHG signal mean intensity with CDA/Ga. These data indicated that higher amounts of fibrillar collagen were synthesized when the reconstruction occurred in the presence of Ga. Lastly, three-dimensional micro-computed tomography was used to quantify total defect filling (Figure 6C). We found that Ga induced a significant 23% increase in defect filling (53.51% vs. 76.89%). Taken together, our results indicated that the Ga presence within CDA used for bone defect filling led to a significantly enhanced bone reconstruction.

**Discussion**

*In vitro*, using CPC and CPC/Ga as growing surfaces, primary human monocytes do not differentiate into osteoclasts when cultured in the presence of M-CSF/
RANKL, whereas successful differentiation was achieved on a plastic surface. Thus, we conclude that the monoculture of primary human monocytes on CPC and CPC/Ga pellets is not a suitable model to study the possible impact of Ga on osteoclastogenesis in vitro. In a previous publication, characterizing the properties of cements used in the present study, an increase in the Ga release was observed over time in the presence of RAW264.7 cells seeded on the surface of cement pellets and stimulated with 20 nM RANKL (Mellier et al., 2015). It should be noted that, compared with the human primary cells used for the present study, RAW264.7 is a mouse cell line that required only the presence of RANKL to differentiate into osteoclasts. We can hypothesize that human primary monocytes are more demanding in terms of environment to initiate osteoclastic differentiation. Indeed, in the absence of any exogenous effectors, co-culture with human primary osteoblasts is required to provide all necessary triggers for successful osteoclastogenesis. In these conditions, optimal interactions occurred through a cell-to-cell contact mediated at least by RANKL attached at the surface of osteoblasts and RANK that is expressed at the surface of osteoclasts (Walsh and Choi, 2014).

In co-culture experiments, it is difficult to comment on the Ga impact on marker genes whose expression was also found in osteoblasts alone. Osteoblastic expression has been reported for transcription factors NFATC1 and AP1 family members (Fos/Jun) (Koga et al., 2005; Komori, 2006), as well as for ITGB3 and CTSK (Grzesik and Robey, 1994; Mandelin et al., 2006). However, regarding genes restricted to osteoclasts such as ACP5 (also known as TRAP) and MMP9, Ga exerts an unambiguous and pronounced inhibitory action. Interestingly, Ga impact on ACP5 expression was further supported at the protein level, as attested by ACP5 histochemical staining presented in Figure 4. Lastly, we did not observe any Ga-induced variation in RANKL, MCSF or OPG gene expression, suggesting that the Ga inhibition of osteoclasts is not mediated through a modulation of the RANKL/OPG ratio.

When it comes to bone-forming osteoblasts, although we observed in vitro a decrease in human primary osteoblasts viability/number on CPC/Ga compared with the control, we evidenced in vivo in a rat femoral defect a 23% (total defect filling) and a 74% (fibrillar collagen) positive impact on bone tissue reconstruction. This can be linked to the dual effect Ga exerts, i.e. a decrease in osteoclastogenesis together with an increased osteoblastic production of factors favouring bone construction. In one previous study, using the murine MC3T3-E1 osteoblast precursor cell line, we showed that Ga treatment enhanced the expression of marker genes including alkaline phosphatase (ALpl), osterix (Osx) and runt-related transcription factor 2 (Runx2) (Verron et al., 2010b), thus promoting osteoblastogenesis. In the present study, however, mature differentiated human primary osteoblasts were used and we observed that Ga downregulates the expression of early osteoblastogenic marker RUNX2, whereas the expression of alkaline phosphatase (ALPL), a by-product of osteoblast activity, remains intact at day 7 of cell culture. On the other hand, Ga stimulated COLIA1 expression, which importantly contributes to new tissue formation as the most abundant bone extracellular matrix protein (Gelse et al., 2003). Similarly, Rockman et al. (1993) reported that Ga increased type I collagen and fibronectin mRNA, as well as collagen protein levels in primary cultures of rat calvarial osteoblasts. The same year, Jenis et al. (1993) published results indicating that Ga increased basal type I collagen mRNA levels and reduced basal osteocalcin mRNA levels in normal rat osteoblasts in vitro. We also observed that the expression of genes encoding for non-collagenous proteins was enhanced, including IBSP, ON and POSTN. Bone sialoprotein II (IBSP or BSP II) is a phosphorylated glycoprotein that is involved in the initial steps of bone tissue mineralization (Bouleffour et al., 2014; Chen et al., 1992). Osteonectin (ON; or SPARC for ‘secreted protein, acidic and rich in cysteine’) is also linked to tissue mineralization along development, remodelling and bone repair (Ribeiro et al., 2014). Regarding periostin (POSTN, originally known as ‘osteoblast-specific factor’ or OSF2), this glutamate-containing protein is preferentially located in the periosteam, and its expression is enhanced during bone fracture repair (Merle and Garnero, 2012). The abovementioned non-collagenous proteins are mainly expressed in the bone matrix mineralization phase, thus they characterize mature active osteoblasts. Finally, Ga downregulated the expression of osteopontin (OPN), a calcium-binding protein that participates in the regulation of mineral crystal growth but also in anchoring osteoclasts to the bone matrix (Liu et al., 2015) and in the formation of their ruffled resorptive borders (Franzen et al., 2008). In addition to the well-described Ga inhibitory action on osteoclastogenesis, our data support the hypothesis that Ga-induced enhancement of bone repair measured in vivo could also be linked to an enhancement of the expression of mature osteoblast genes involved in bone tissue formation.

In a cylindrical femoral defect, Ga delivered via CPC failed to show any impact on bone reconstruction within the defect. This is probably due to the low Ga quantities released from the apatite matrix and available in the surrounding tissue. On the contrary, using CDA we demonstrated that the presence of Ga induced a significant stimulation of bone reconstruction, as attested by two types of quantification, i.e. three-dimensional micro-computed tomography scan and SHG analysis. In 1993, Jenis et al. reported that systemic Ga administration decreased serum calcium levels and reduced osteocalcin steady-state mRNA levels, while having no effect on type I collagen mRNA levels or bone mineral density in vivo. The comparison with these results from Jenis et al. (1993) points out how crucial the delivery mode of a pharmacological agent can be. Indeed, using systemic administration of a therapeutic dosage used for the treatment of Paget’s disease, they did not observe any impact on bone mineral density of the left femur in normal rats treated with Ga, whereas we measured a positive effect via Ga-loaded biomaterial microparticle implantation. This discrepancy could be related to the
local delivery mode of Ga we used, leading to higher local concentrations of an active molecule released continuously. In addition, and these hypotheses are not exclusive, Jenis et al. (1993) reported a Ga effect on normal bone remodelling, whereas we addressed the issue of bone reconstruction following the creation of a defect. Thus, these different physiological situations could also account for our respective observations.

Regarding SHG, these results are in agreement with the data we obtained measuring COL1A1 gene expression. Actually, SHG analysis allows the quantification of fibrillar collagen (Figure 6B) and we also observed that Ga stimulated COL1A1 gene expression in primary osteoblasts (Figure 3C). Together, these data reinforce the correlation reconstruction we measured in vivo. Importantly, it should be stressed that to our knowledge, this is the first time that the use of SHG is reported in the field of bone reconstruction quantification within a bone substitute implanted in vivo. Indeed, concerning osteo-articular applications, SHG analysis has been used to evaluate osteogenic cell differentiation in vitro (Brackmann et al., 2012; Flausse et al., 2013; Hronik-Tupaj et al., 2011; Lee et al., 2006; Liskova et al., 2015) or to study tissue samples retrieved from normal or pathological situations (Adur et al., 2013; Ambekar et al., 2012; Caetano-Lopes et al., 2009, 2010; Huang et al., 2015; Masago et al., 2012; Nadiarnykh et al., 2007; Paietta et al., 2013; Reiser et al., 2007; Tang et al., 2015). Based on the consistency we observe with gene expression and three-dimensional micro-computed tomography scan quantification data, we demonstrate in the present report that SHG analysis is also fully suitable to monitor the reconstructive properties of a bone substitute upon its implantation in vivo.

In conclusion, we propose that the Ga positive impact reported in this study is linked to a decreased osteoclastic differentiation coupled with an increased new bone tissue formation. In terms of mechanisms, this is understated by in vitro data obtained at the molecular level through gene expression analysis. In vivo, Ga enhanced CaP-induced bone reconstruction, as observed using CDA/Ga to fill bone defects generated in a rat model. Thus, we suggest that the presence of Ga could be beneficial to the process of bone regeneration, especially in pathological situations related to excessive bone resorption. As a future development, it will be interesting to assess Ga-loaded biomaterial performance in the context of osteoporotic bone. Due to their mechanical properties and in vivo resorption rates, we consider that CaP bone substitutes constitute attractive vectors for local Ga delivery to bone.

Interestingly, in addition to the positive impact on bone formation reported here, Ga offers other worthwhile prospects. Indeed, as mentioned in the Introduction section above, Ga displays anti-neoplastic, anti-infectious and anti-inflammatory properties (Chitambar, 2010). Further studies are required to fully evaluate the potentialities of Ga-loaded bone substitutes used for the filling of bone defects for which tumour development and infections represent critical issues. Finally, as a beneficial side-effect of our quantification experiments, we provide strong data supporting the use of the SHG technique to address the issue of in vivo tissue reconstruction within a bone substitute.

Conflict of interest

Ivana Strazic Geljic, Florian Boukhechba, Charlotte Mellier, Pascal Janvier, Jean-Michel Bouler, Elise Verron and Jean-Claude Scimeca received research support from Graftys SA. The terms of these arrangements have been reviewed and approved by CNRS, the University of Nice Sophia Antipolis and the University of Nantes, in accordance with their policy on objectivity in research.

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References


Chitambar CR. 2012; Gallium inhibits bone marrow stem cell morphogenesis in polyglycolic acid scaffold by multiphoton microscopy. Tissue Eng Int 12: 2835–2841.


Ma Z, Fu Q. 2010b; Therapeutic effect of organic gallium on osteocondensed osteosarcoma rats by decreased serum minerals and increased bone mineral content. Biochem Biophys Res Commun 398: 495–496.


Huang C, Ness VP, Yang X, et al. 2015; Spari-temporal proteomic analysis of different human bone tissues reveals a unique chondrocyte phenotype in the deep look: modern microscopy technologies to optimize the design and functionality of biocompatible scaffolds for tissue engineering in regenerative medicine. J R Soc Interface 10: 20130263.


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