Ectopic bone formation using an injectable biphasic calcium phosphate/Si-HPMC hydrogel composite loaded with undifferentiated bone marrow stromal cells

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Abstract

We have used a new synthetic injectable composite constituted of hydroxyapatite/tricalcium phosphate (HA/TCP) particles in suspension in a self-hardening Si–hydroxypropylmethylcellulose (HPMC) hydrogel. The aim of this study was to evaluate in vivo the biocompatibility and the new bone formation efficacy of this scaffold loaded with undifferentiated bone marrow cells (BMSCs). This biomaterial was mixed extemporaneously with BMSCs prepared from C57BL/6 mice, injected in subcutaneous and intramuscular sites and retrieved 4 and 8 weeks after implantation. Dissection of the implants revealed a hard consistency and the absence of a fibrous capsule reflecting a good integration into the host tissues. Histological analysis showed mineralized woven bone in the granule inter-space with numerous active osteoclasts attached to the particles as assessed by the presence of multinucleated cells positively stained for TRAP activity and for the a3 subunit of the V-ATPase. Small vessels were homogenously distributed in the whole implants. Similar results were obtained in SC and IM sites and no bone formation was observed in the control groups when cell-free and particle-free transplants were injected. These results indicate that this injectable biphasic calcium phosphate–hydrogel composite mixed with undifferentiated BMSCs is a new promising osteoinductive bone substitute. It also provides with an original in vivo model of osteoclast differentiation and function.

Keywords: Bone induction; Injectable biomaterial; Si–HPMC/BCP composite; Bone marrow stromal cells; Mouse

1. Introduction

A major challenge in bone regeneration is the development of efficient delivery approaches for cells involved in this process. Following expansion in tissue culture, bone marrow stromal cells (BMSCs) from various species have been combined with scaffolds and transplanted into heterotopic sites or in bone defects in animal models to improve new bone formation [1–3]. In human, these cells have been used in two reports of long bone reconstruction after segmental defect [4,5]. A lot of studies have demonstrated that a major difficulty remains the low efficiency of cell seeding. The cells are found close to the surface but not in the core of the scaffolds and the bone induced after implantation is usually formed in the peripheral pores of the scaffolds [6]. Many approaches are currently under investigation to improve the cellular colonization before implantation such as the development of bioreactors for cell seeding of compact scaffolds.
as well as the use of granular instead of compact substitutes. In this line Mankani and coworkers have observed more extensive bone formation with BMSCs attached to hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder than to HA/TCP blocks [13]. A common feature of these studies is that the preparation of the transplants requires an in vitro culture period allowing cell adhesion to the biomaterial. The development of gel-like polymers offers the opportunity to create new composites that can be more easily invaded by cells or in which the appropriate cells can be incorporated before implantation. Synthetic hydrogels in development include poly(propylene fumarate-co-ethylene glycol) [14]. PEG hydrogels [15], polyethylene oxide [16], sodium hyaluronate [17], sodium alginate [18], self-assembling peptide hydrogels [19] and hydroxypropylmethylcellulose (HPMC) [20]. HPMC polymers/biphasic calcium phosphate composites have been successfully developed by Daculsi and coworkers as a ready to use injectable bone substitute (IBS) [20–24]. This first generation of IBS composite, which consists of a water soluble 3% cellulose polymer and biphasic calcium phosphate (BCP) particles, is a non-hardening material. In order to improve the physical properties of this IBS, silane was grafted to the HPMC [25,26]. In our previous work, the hardening time (10 min) and consistency of this Si–HPMC hydrogel have been set up to make it easy to manipulate in vitro and to inject in vivo [27]. In these conditions this Si–HPMC hydrogel has been shown to be biocompatible in vitro and a well-adapted matrix for 3D culture and differentiation of osteoblastic [27] and chondrocytic [28] cells. In the present work, this Si–HPMC hydrogel has been mixed with calibrated BCP particles (60% hydroxyapatite and 40% β-tricalcium phosphate) to obtain a synthetic composite that will be abbreviated as IBS2 in the body of the text. We have loaded IBS2 with amplified mouse bone marrow stromal cells and tested its biocompatibility and bone tissue induction properties after subcutaneous (SC) and intramuscular (IM) injections in C57BL/6 mice. These implants have been compared to cell-free and particle-free transplants.

2. Materials and methods

2.1. Biomaterial

IBS2 is an injectable biomaterial composed of a slanted HPMC (Si–HPMC) associated with biphasic (60% HA and 40% TCP) CaP ceramic particles calibrated between 40 and 80 μm [25,26]. Si–HPMC polymer was provided by Dr. Pierre Weiss and CBP ceramic particles by Biomatlante (Vigneux de Bretagne, France). Briefly, the Si–HPMC hydrogel results from the mix, at room temperature, of a 3% (w/v) Si–HPMC-polymer solution (pH 12.9) with a buffered culture medium (pH 3.6) in a 2/1 ratio (v/v) as described previously [27]. The buffered medium is composed of alpha-MEM medium concentrated 1.4× (prepared from the powder, Invitrogen, Cergy Pontoise, France), supplemented with NaHCO3 0.26 mM (Invitrogen) and Hepes 0.13 M (Sigma–Aldrich, L’Isle d’Abeau Chesnes, France), and adjusted to pH 3.6 with HCl. IBS2 results from the mix of this Si–HPMC hydrogel with BCP particles at various BCP/hydrogel (w/v) ratios. In the present work, we used a 40% ratio. This biomaterial reticulates at room temperature within 10 min in a gelatin state at pH 8 without exothermic reaction.

2.2. Preparation of bone marrow stromal cells from C57BL/6 mice and cell incorporation into IBS2

BMSC were prepared from C57BL/6 mice (Janvier, Le Genest St Isle, France) bone marrow. Briefly, six long bones per mouse (two each of femur, tibia and humerus) were aseptically removed and the entire bone marrow content of medullary cavities was flushed with complete medium made of alpha-modified minimum essential medium (αMEM, Cambrex, Emerainville, France) supplemented with 10% Hyclone fetal calf serum (FCS) (Perbio, Bezons, France), 2 mM t-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Single cell suspensions of marrow cells extracted from each mouse which contained 5–6 × 10^6 nucleated cells were plated in 3 × 100 mm culture petri dishes in complete medium in the absence of additional differentiating factors and cultured at 37 °C, 5% CO2 in humid atmosphere. Medium was changed twice a week. Confluent layers formed after 11–14 days were harvested by trypsination and counted. The viability of the cells was evaluated using the trypan blue exclusion method. These cells were washed three times in PBS, suspended in PBS containing 10% of C57BL/6 mouse serum and mixed at final concentration of 2 × 10^6 per 100 μl either into IBS2 (Si–HPMC + BCP) or into Si–HPMC hydrogel alone.

2.3. Cell viability into IBS2

The viability of the BMSCs incorporated into Si–HPMC hydrogel or IBS2 was assessed using the LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Invitrogen, France) as previously described [27]. Briefly, samples of hydrogel or IBS2 containing BMSCs were washed with PBS and stained for 30 min at room temperature with PBS containing 2 μmol/l calcein-AM and 4 μmol/l ethidium homodimer-1. Calcein-AM is a non-fluorescent cell-permeant fluorescein derivative, which is converted by cellular esterase activity into cell-impermeant and highly fluorescent calcein. Calcein accumulates inside live cells having intact membranes and results in a green fluorescent signal. Ethidium-homodimer-1 enters dead cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to their DNA leading to a red fluorescent signal. After 30 min incubation at room temperature, samples of hydrogel or IBS2 containing BMSCs were dropped onto a glass slide, covered with a coverslip and viewed under an Axioskop fluorescence microscope (Carl Zeiss, Le Pecq, France) equipped with FITC and Texas Red filters. The number of live and dead cells was estimated using the Challengar Gold Master software.

2.4. Mouse implantation

These experiments were conducted according to the guidelines of the Direction des Services Vétérinaires and received the approval of the local animal care committee. A total of 30 C57BL/6 mice of 8 weeks old were anesthetized by 4% isoflurane inhalation. IBS2 loaded with BMSCs as described above was transferred into a 1 ml syringe mounted with a 18G needle and injected percutaneously. Two injections of 80 μl were performed for each mouse: one subcutaneous (SC) beneath the dorsal skin between the scapulae and one intramuscular (IM) in the hind leg. Three experimental conditions were used (ten mice per group): (1) Si–HPMC hydrogel without BCP particles delivering BMSCs, (2) cell-free IBS2, (3) IBS2 delivering BMSCs. At 4 and 8 weeks, five animals of each group were sacrificed with carbon dioxide and the implants were retrieved.

2.5. Histological analysis

Implants with surrounding tissues were dissected and fixed in 10% buffered formalin for 24 h. Each implant was sectioned in two to three pieces through its midline and embedded in paraffin. Serial sections of

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4 μm were deparaffinized, hydrated and stained with haematoxylin, erythrosin and safran (HES). Photomicrographs of internal sections of each sample were taken with a Zeiss Axioskop light microscope and a JVC color video camera KY-FS90 3CCD. Images were then analyzed using Challenger/Clara Vision Software.

Neovascularization and osteoclast number were assessed by counting, respectively, the capillaries and the multinucleated cells under light microscopy by two different observers (× 400). Osteoclasts and vessels density were expressed by mm² as the mean ± standard deviation. All data were analyzed with Statview 5.0. The statistical test used was the Fischer exact test. A p-value under 0.05 was considered significant.

2.6. Goldner staining

Paraffin-embedded sections of 7 μm were stained with Goldner’s trichrome method to detect bone and osteoid tissues. This method results in green staining for the mineralized bone and red for unmineralized matrix-ossteoid. Briefly, deparaffinized sections were rehydrated and incubated in Weigert’s iron haematoxylin for 20 min, washed in tap water and differentiated in 1% acid alcohol, washed in tap water 5 min and rinsed in distilled water. Sections were then stained in Ponceau/acid fushin/azophloxine/acetic acid for 5 min, rinsed with 1% aqueous acetic acid, treated with Orange G/phosphomolybdic acid for 20 min, rinsed in 1% aqueous acetic acid, stained in light green/acetic acid for 5 min, washed in aqueous acetic acid for 5 min and blot dry. Pertex (Microm France, Francheville, France) mounting was used.

2.7. Tartrate-Resistant Acid Phosphatase (TRAP) staining

Explanted samples were embedded in cryomatrix (Thermo Shandon, Pittsburgh, USA) and frozen by immersion in cold isopentane. Sections of 5–7 μm were cut with a cryotome (Thermo Shandon), placed on polylysine-coated glass slides and dried. TRAP activity was analyzed using the TRAP Kit 386A (Sigma–Aldrich, L’Isle d’Abeau Chesnes, France) according to the manufacturer’s instructions. TRAP positive cells appeared in red with nuclei in blue.

2.8. Immunohistochemistry of the α3 subunit of the V-ATPase

The anti-α3 rabbit polyclonal antibody was raised against a peptide specific for the α3 subunit of the V-ATPase localized in the N-terminal part of the mouse protein (103-RLAQELRDVRGNQQA-117, Genbank Q13488). Briefly, 5-μm-thick formalin-fixed frozen tissue sections were fixed 10 min with ice-cold acetone, washed in PBS and incubated 30 min in 0.3% H₂O₂ diluted in methanol. After two washes in PBS, the slides were incubated in 1.5% normal goat serum (blocking buffer) for 1 h then with primary antibody (pre-immune and immune serum) at 1/100 dilution in blocking buffer for 30 min. After washing in PBS, biotinylated anti-rabbit antibody incubation and immunoperoxidase staining procedure were performed using the rabbit ABC staining system (sc-2118, Santa Cruz, CA, USA) following the manufacturer’s protocol. Sections were incubated with peroxidase substrate for 10 min and counterstained in haematoxylin for 3 min. Following dehydration steps, pertex mounting medium was used (Microm France).

3. Results

3.1. Cytological examination of the cells after loading into IBS2

The viability of the BMSCs freshly incorporated into IBS2 was analyzed before injection using the LIVE/DEAD Viability/Cytotoxicity test. As shown in Fig. 1, the BCP particles were homogenously distributed into the bioma-
implants were free of cells and appeared as a transparent area. After 8 weeks, histological aspect of the implants were identical but the peripheral crown was thinner than that observed at 4 weeks (data not shown).

Dissection of cell-free IBS2 implants after 4 weeks revealed an elastic consistency and histological analysis showed a homogenous distribution of BCP particles within the implants (Fig. 2B). In this group, all implants were free of cells except in a peripheral border of 50–200 μm maximum. This thin zone consisted in a polymorphic cellular reaction with a fibroblastic proliferation, many monocytes, granulocytes and macrophages. Multinucleated macrophages were observed surrounding the BCP particles and numerous small vessels were present. After 8 weeks implant consistency remained elastic on palpation and similar cellular reactions were observed in the peripheral crown, which was again thinner than that observed at 4 weeks (not shown).

Fig. 2. Histological analysis (HES staining) after 4 weeks of (A) hydrogel loaded with BMSCs and of (B) cell-free IBS2 with BCP particles appearing black (scale bar: 1 mm).

Fig. 3. Bone tissue formation by BMSCs incorporated into IBS2. Representative HES stained cross-sections of BMSCs/IBS2 implants (A) injected ectopically in C57BL/6 mice and harvested after 4 weeks; (B) higher magnification showing woven bone with osteocytes (white arrows) and osteoclasts (black arrows); higher magnifications showing (C) blood vessels (white arrows) and (D) osteoblasts (black arrows) and osteocytes (white arrows) (scale bars—A: 1 mm; B: 100 μm; C and D: 50 μm).
BMSCs/IBS2 constructs retrieved after 4 and 8 weeks had a hard consistency. After 4 weeks, four implants out of ten were fully colonized with woven bone in contact with the ceramic in all the intergranular spaces (Figs. 3(A) and (B)). Numerous multinucleated cells were attached to the BCP particles (Fig. 3B) and many blood vessels were homogenously distributed in the center as well as in the periphery of the implants (Fig. 3C). We observed active cubic osteoblasts forming columnar layers on the surface of the BCP particles (Fig. 3D) and osteocytes embedded in the matrix structure (Figs. 3(B) and (D)). Numeration revealed an average of $27.4 \pm 0.5$ multinucleated cells/mm$^2$ and $15.4 \pm 4.8$ vessel/mm$^2$ in the 4 weeks implants. We often observed in the periphery of the implants a thin zone rich in granulocytes. In the other six implants, 80% of the volume was colonized by woven bone, vessels and multinucleated cells. The remnant central part was not invaded with new bone but BCP particles were surrounded by numerous lymphocytes and macrophages (data not shown).

After 8 weeks, seven implants out of ten were full of new bone. In these implants, the tissue had the same characteristics as observed at 4 weeks. Numeration revealed an average of $30.2 \pm 5.2$ multinucleated cells/mm$^2$ and $18.2 \pm 3.5$ vessel/mm$^2$. No statistical difference was found between cell and vessel numerations of the 4 and 8 week implants. In the other three implants, we observed a progression of the woven bone invasion and the central area free of tissue was reduced compared to that observed at 4 weeks. All these implants were positive for Goldner staining at 4 and 8 weeks indicating that this new tissue was mineralized (Fig. 4).

### 3.4. Characterization of multinucleated cells

The presence of multinucleated cells stained positively for TRAP activity strongly suggested that these cells attached to the BCP particles were osteoclasts (Fig. 5). The immunological detection of the a3 subunit of V-ATPase at the plasma membrane (Fig. 6A, B) and more specifically at the membrane portion in contact with the particles (Fig. 6C) is a strong argument in favor of a resorbing activity of these cells. This was supported by the observation of micro-particles present in the cytoplasm of these cells in both 4 and 8 weeks implants (data not shown).

### 4. Discussion

IBS2, a new composite made of BCP particles of 40–80 μm in suspension into a Si–HPMC hydrogel, is obtained by mixing a 3% Si–HPMC viscous polymer with a buffered medium and BCP particles in defined proportions. It can be seeded with cells and injected using an 18G needle within 10 min after mixing. In these conditions the SC and IM grafts maintained their 3D form and location. We have also observed that mouse BMSCs seeded into IBS2 can be maintained in vitro in 3D culture with an excellent viability for at least 3 weeks (data not shown). This is consistent with our previous data showing that human osteogenic cells can be cultured in 3D into Si–HPMC hydrogel for at least 3 weeks [27]. IBS2 combines the advantages of two different phases. The hydrophilic polymeric phase makes this composite injectable, serves as space-holder to prevent granule packing and also provides with a vehicle for homogenous delivery of cells into the graft site. In this phase, we have shown previously in vitro that single-cell suspension of normal human osteogenic cells survive during several weeks but do not proliferate [27]. This emphasizes the importance of the HA/TCP ceramic phase of IBS2 as a support for attachment, proliferation and differentiation of stem cells.
and progenitors as described for other BCP scaffolds [29,30].

The absence of fibrosis encapsulation demonstrated the good integration of the composite into the host surrounding tissue. Our study shows that undifferentiated mouse BMSCs loaded in IBS2 and injected SC and IM, leads to the formation of woven bone with marked invasion by numerous osteoblasts, new vessels and active osteoclasts. The similarity of results obtained in SC and IM sites are consistent with other recent data obtained with rat stromal cells seeded on titanium fiber mesh implants, which have shown no significant differences in bone amount between these two sites [31]. In some IBS2/BMSCs implants, a small central area remained free of new bone but a marked progression of new tissue invasion was clearly observed after 8 weeks compared to 4 weeks. Comparatively, no bone formation was observed in cell free or BCP free implants. The absence of bone induction in cell-free IBS2 implants demonstrates that the incorporation of BMSCs is responsible for the formation of new bone which appear to progress in a centripetal direction. Whether these putative progenitor cells actually contribute to the process of skeletal regeneration themselves, or simply act as a source of growth factors or cytokines is currently under investigation.

Our results can be compared with the ones obtained by Mankani and coworkers who have used HA/TCP particles of various size combined with human BMSCs and fibrin glue to implant immunocompromized Bg-Nu-XID mice. These authors have shown that HA/TCP particle size plays a crucial role in determining the extent of bone formation, the best amount of bone being obtained when using particles of 100–250\,\mu m and the lowest amount obtained with particles below 44\,\mu m. The authors hypothesized that the absence of bone formation with the smallest particles could be linked to the impairment of vascularization due to the close packing of these particles [13]. Our results differ from those since we have found that IBS2 containing particles of 40–80\,\mu m associated with mouse BMSCs leads to the formation of a vascularized new bone. Besides

Fig. 6. Immunohistochemistry of the a3 subunit of the V-ATPase on frozen tissue sections of 4 weeks IBS2/BMSC implants: (A) non-immune serum hybridization showing nuclei colored in blue; (B) immune serum hybridization showing brown staining of a3 subunit; (C) higher magnification showing a3 subunit staining (brown) located at the osteoclast membrane portion in contact with BCP particles (scale bars: A, B:50\,\mu m; C:10\,\mu m).
variability between human and mouse species [32], this
difference could result from the presence of the Si–HPMC
hydrogel, which might help maintaining space between the
particles and preventing packing. This inter-particle space
favors vascular and cell colonization as well as transmis-
sion of body fluid pressure and free diffusion of nutrients.
The choice of the granule size used in the present work was
based on previous results obtained with the first generation
of IBS, which have shown that BCP grains of 40–80 and
80–200 μm supported similar bone ingrowth after injection
into femoral defect [24].

Our data can also be compared with those of Yamada
and coworkers who described a combination of fibrin glue,
βTCP and differentiated MSC which results in successful
bone formation after SC injection in rats [33]. These results
were obtained after a 20 day culture period of rat BMSCs
in differentiation medium on disc-shaped blocs reduced in
small lumps before injection. Comparatively we show here
that IBS2 offers the possibility of an extemporaneous
incorporation of cells without pre-adhesion to the BCP
granules as also required with most of the scaffolds. We
show here that bone invasion was obtained only when IBS2
was loaded with BMSCs and that no bone was observed
with BMSCs loaded into BCP-free implants. Moreover
BMSCs were amplified in the absence of differentiating
agents. These results strongly suggest that BCP ceramic
particles associated with Si–HPMC hydrogel have osteoin-
ductive properties by driving these mesenchymal precurs-
orers into the osteoblastic lineage, even in non-bony sites.
Several mechanisms can be proposed to explain the
osteoinduction promoted by BCP. In addition to providing
with an adhesion phase for cell growth and differentiation,
the efficiency of BCP is related to its capacity for partial
dissolution due to the bioactive stability of HA and the
bioactive solubility of TCP as described by Daculsi and
coworkers. The microporosity of BCP particles is necessary
bioactive solubility of TCP as described by Daculsi and
coworkers. The microporosity of BCP particles is necessary
for fluid circulation and to induce the dissolution/precipitation
process for CaP crystals [34,35].

We have observed an early invasion of IBS2/BMSCs
implants with numerous multinucleated TRAP positive
and a3 subunit positive osteoclasts (OC). These cells were
present homogenously throughout the grafts and always in
contact with BCP particles. The positive expression of the
a3 subunit of the V-ATPase at the plasma membrane in
contact with the BCP strongly suggests that these OC were
functional. Indeed, the expression of the a3 subunit of the
V-ATPase increases during osteoclast differentiation and
this expression allows the V-ATPase to be addressed to the
plasma membrane [36,37]. The presence of the V-ATPase
at the membrane leads to the production of protons and to
bone mineral resorption. Scaffold degradation rate in bone
tissue engineering seems a critical design parameter for
bone regeneration [38,39]. Previous results obtained
with BCP ceramics in vitro [40] and the present data
suggest that osteoclast activity plays a major role in the
biodegradation of the BCP material in vivo. This resorp-
tion phase is likely an important step in the subsequent
development of new bone within IBS2, as observed for
normal bone development. The numerous osteoclasts
present into IBS2/BMSCs implants likely originates from
mononuclear myeloid precursors brought to the BCP
particles through new vessel formation or migration from
the microenvironment. Formation of these multinucleated
cells seems to be associated with the presence of the BCP
particles since they were also present in the peripheral zone
of the cell-free IBS2 implants. Conversely, in BCP-free
implants, numerous mononuclear macrophages but no
multinucleated cells were observed. Several mechanisms
could be involved in macrophage attraction and OC
differentiation induced by BCP such as the pro-inflamma-
tory reaction induced by BCP as already described [41] and
the adhesion surface allowing attachment and fusion of the
mononuclear phagocytes. Based on these results, IBS2/
BMSCs provides with an original in vivo model of
osteoelastic differentiation.

Besides their crucial role in graft survival, the numerous
vessels that we observed in the IBS2/BMSCs implants
might be also involved in the new tissue formation. Several
studies have demonstrated the major role of angiogenesis
in bone formation and bone remodeling [42–45]. Our
results demonstrate that both BMSCs and BCP are
required for the development and invasion of new vessels
in the whole implants. The role of BCP particles is unclear
but could be linked to the inflammatory reaction triggered
by the BCP ceramic [41], which involves neoangiogenesis.
In this line, macrophages–osteoclasts attracted into the
implants probably secrete a broad array of cytokines and
growth factors facilitating vascular development including
vascular endothelial growth factor (VEGF). BMSCs might
also play an important role through the secretion of
angiogenic cytokines as suggested by Pinney and colleagues
who have shown that VEGF mRNA level was 22-fold
greater in 3D versus 2D fibroblast cultures [46]. Ito and
coworkers have shown recently that VEGF and RANKL
signals, which are known to dominantly regulate angiogen-
esis and osteoclastic bone resorption, are necessary and
sufficient for efficient allograft remodeling [47]. Finally, we
believe that, in IBS2 implants loaded with BMSCs, the
coexisting presence of mineralized woven bone, osteo-
blasts, osteocytes and functional osteoclasts are strong
arguments for bone remodeling process.

5. Conclusion

We show here that this injectable biphasic calcium
phosphate/Si–HPMC hydrogel composite, loaded with
undifferentiated BMSCs has osteoinductive properties in
ectopic sites. Based on the colonization of these implants
with a large number of osteoblasts, osteocytes, functional
osteoclasts and with mineralized woven bone, one can
predict that an efficient bone remodeling should take place
also after implantation in bony sites. Therefore, this
injectable biomaterial is a good candidate for bone tissue
engineering. It also provides with an original in vivo model to explore osteoclastic differentiation and function. 

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