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Inflammatory reaction in rats muscle after implantation of biphasic calcium phosphate micro particles

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Abstract Several studies have shown that macro micro porous bioceramics ectopically implanted promote bone tissue formation. This study aims at investigating the inflammatory response towards biphasic calcium phosphate (BCP) ceramic micro particles. BCP composed of hydroxyapatite (HA) and beta-tricalcium phosphate, HA/β-TCP ratio of 50/50, were prepared by sintering at 1200°C for 5 h. After crushing, 3 fractions of BCP micro particles <20, 40–80 and 80–200 μm were sieved. The micro particles were carefully characterized by using X-ray diffraction (XRD), scanning electron microscopy (SEM) and laser scattering. The inflammatory reactions induced by BCP micro particles implanted in quadriceps muscles of rats for 7, 14 and 21 days were studied by histology ($n = 8$/group). A fibrous tissue encapsulation of the BCP micro particles implanted in muscle tissue was observed and fibrosis was similar for the 3 groups of micro particles. The comparison of the cellular response indicated that the total number of cells was significantly higher for BCP <20 μm than for 40–80 and 80–200 μm ($p < 0.0001$). The number of macrophages was relatively higher for the smallest than for the intermediate and largest fractions ($p < 0.0001$). The relative percentage of giant cells was higher for the intermediate and largest size of particles than for the smallest. The number of lymphocytes was comparable for the 3 fractions and after the 3 delays. Therefore, the BCP micro particles <20 μm initiated an inflammatory response which might play an important role in osteogenesis.

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1 Introduction

The current challenge in orthopaedic and maxillofacial surgeries is the reconstruction of large bone defects. Autologous bone graft is considered as the gold standard in bone reconstruction because it has osteogenic, osteoinductive, osteoconductive and resorbable properties [1–3]. Nevertheless, autologous bone graft must be harvested from another surgical site (e.g. iliac crest) prior to fill a bone defect. It is therefore limited in quantity, less than 20 cm^3, and often associated with complications at the harvesting site. Calcium phosphate bioceramics have been prepared in different forms such as powders, granules, macroporous blocks for filling bone defects. During the last two decades, Hydroxyapatite (HA), β-tricalcium phosphate (β-TCP) and biphasic calcium phosphate (BCP) bioceramics have been widely used in orthopaedic, maxillofacial and dental surgery [4, 5]. Bioceramics have biocompatible, bioactive and osteoconductive properties [6, 7]. However, these synthetic materials generally lack osteogenic and osteoinductive properties for regenerating large amounts of bone tissue. Several studies have recently reported the induction of bone tissue by certain bioceramics implanted in muscles or subcutis of various animal models [8–11]. After implantation in these ectopic sites for several weeks, mature and mineralized bone tissue formation has been observed inside macro and micro porous ceramics even thought the materials were not combined with osteogenic cells or factors, e.g. bone morphogenetic proteins. Although the precise biological mechanism leading to bone induction by bioceramics remains unknown, several groups have shown that the presence of micropores about 0.1–5 μm in diameter at the surface of ceramics is a prerequisite for ectopic bone formation [11–13]. This microporosity located between ceramic grains is related to relatively low sintering temperature

(900–1100°C) but obviously depends on the starting calcium phosphate powder, manufacturing process and heating/cooling time. Furthermore, the microporosity may influence the material cohesion *in vivo* as the dissolution of grain boundaries would be higher for micro porous than fully sintered materials. This dissolution of grain boundaries in physiological fluids may lead to the release of microparticles at the vicinity of bioceramics [14–16]. These microparticles would then activate macrophages, giant cells and lymphocytes which will release specific cytokines [17–21]. These cues could interact with circulating stem cells or other types for differentiating them into osteoblasts producing bone tissue. This cascade of biological events could be similar to those occurring in the healing of bone fractures. Indeed, micro debris and particles of mineralized bone are often observed inside bone fractures. These micro particles are first degraded by macrophages and osteoclasts prior to the production of a new extracellular bone matrix by osteoprogenitor cells. The relationship between tissue degrading cells like macrophages and osteoclasts and bone forming cells has been subjected of numerous reports [17–23]. These studies have investigated the effect of nano- or micro-sized particules *in vitro* using macrophages and/or osteoblasts on the secretion of several cytokines [22, 23]. It has been found that high amounts of cytokines such as transforming necrosis factor-apha (TNF-α) and interleukin 6 (IL-6) were produced by macrophages in the presence of micro particles [20]. In addition, a decrease in cell viability was observed in contact with micro-particles [14]. Although considerable work has been done *in vitro*, only a limited number of studies have been conducted *in vivo* using micro particles [14, 15]. Malard and collaborators have implanted BCP particles having sizes of 10–20, 80–100 and 200–400 μm in critical sized bone defects of rats for 7, 14 and 21 days. The best bone ingrowth, together with the higher resorption/degradation rate and stronger inflammatory reactions were observed for BCP particles of 10–20 μm than for the other sizes. The authors suggested that a strong, but brief, inflammatory reaction associated with micro particles which implies a massive release of cytokines, was favourable to the bone healing process. Another study conducted with various bone-substitute materials implanted in intraosseous and subcutis of rat demonstrated different biological responses [16]. However, the difference in biological behaviours was mainly related to the chemical composition of the implanted materials and not to their particle size.

The present study aims at investigating the micro environment beside microporous bioceramic particles implanted in muscles of rats. We have investigated the inflammatory reactions induced by BCP micro particles sintered at high temperature (1200°C for 5 h) implanted in muscles of rats for 7, 14 and 21 days. Three sizes of BCP micro particles $<$20, 40–80 and 80–200 μm were carefully characterized by using X-ray diffraction (XRD), Fourier-Transform Infrared spectroscopy, scanning electron microscopy (SEM) and laser scattering. These fractions implanted in quadriceps muscles of rats were then studied by histology in order to determine the cells and tissue reactions.

2 Materials and methods

2.1 Preparation and characterization of BCP micro particles

Calcium deficient apatite (CDA) was prepared by hydrolyzing dicalcium phosphate dehydrate (DCPD) as previously described [24]. After sintering at 1200°C for 5 h, the CDA transformed into a mixture of Hydroxypatite (HA) and beta-tricalcium phosphate (β-TCP) named biphasic calcium phosphate (BCP). The sintered BCP ceramic was crushed and sieved into particle fractions of $<$20 μm, 40–80 μm and 80–200 μm using certified sieves (Fritsch, Analysette, Germany). The 3 fractions were analyzed using powder X-ray diffraction (Philips, PW 1730, France) with a CuKα source operated at 40 kV and 30 mA. The microstructure and particle size distribution of the 3 fractions were observed by scanning electron microscopy (SEM, Leo 1450 VP). For SEM observations, the BCP particles were deposited onto carbon tape and Au-Pd sputtered (Denton Vacuum, Desk III, UK). For the determination of particle size, samples were scanned using backscattered electron and images were analyzed using a semi-automatic image analyser (Quantimet 500, Leica, UK). The size and number of particles were determined using a custom made program. Particle size was also measured using laser scattering (Coulter LS 230, Coultronics, France). Three measurements were performed on each sample. Results were reported as mean diameter (μm) as function of volume (%). After characterization, the 3 fractions were put into glass Pasteur pipettes and sterilized by heating at 200°C for 2 h.

2.2 Animal model and surgical procedure

All animals handling and surgical procedures were conducted according to the European Community guidelines for the care and use of laboratory animals (DE 86/609/CEE). The study protocol was submitted for approval to the local animal care and safety committee. The experiments were performed according to Good Laboratory Practices (GLP) at the Faculty of Medicine of Nantes within approved experimental facilities. Experiments were performed using 36 Wistar Han rats, males, 7 weeks old, weighting 250–275 g (Charles River, France). Rats were housed (four per cage), with water and food at will. The animals were placed in quarantine for at least 10 days prior to surgery. Rats were under general anaesthesia with a halogenous compound, oxygen/Isoflurane (Forane®,

Abbott laboratories, UK). After shaving and disinfection of both legs, a lateral skin incision was made according to the axis of the femur. An intramuscular pocket was created in the quadriceps muscle using blunt dissection. About 0.3 g of BCP micro particles were put into the muscle pocket. Muscle and skin were sutured in layers using resorbable sutured (Vicryl 3-0, Ethicon, France). The same procedure was applied bilaterally thus, giving 2 implants per animal. After surgery, no antibiotic treatment was administrated. Animals were sacrificed 7, 14 and 21 days after surgery using an intra cardiac over dose of sodium pentobarbital (Dolethal, Vetoquinol, France). Samples were retrieved and immediately fixed in neutral formol (10%) for 24 h. Samples were then decalcified, dehydrated and embedded in paraffin. Thin sections (approx. 7 μm) were made in the middle of the sample using microtome (Microm HM355). Hemalun-Pholoxine-Safran (HPS) staining was performed on the sample slices using an automated coloration apparatus. All groups were analysed under light microscopy (Olympus Bx 50). Each slice was divided into 10 fields and cell counting was performed by an anatomopathologist (Nicolas Josselin, MD). The number of giant cells, macrophages and lymphocytes was determined. The thickness of the fibrous tissue layer around the implanted BCP particles was semi-quantitatively analyzed using a score index of 0-2; with score 2 corresponding to a thick fibrous capsule. The fibrosis was an indicator for the persistence of the immune response. Two measures were performed on each sample.

2.3 Statistical analysis

All data were expressed as average ± standard deviation and examined using one-way analysis of variance ANOVA using the XL Stat 6.0 software. The results were considered significant for p values <0.05.

3 Results

3.1 Physicochemical characterization of the BCP particles

Three fractions of BCP particles, <20 μm, 40–80 μm and 80–200 μm, were prepared and characterized. As shown in Table 1, the BCP fractions were solely composed of HA and β-TCP phases in a weight ratio of approximately 50/50 giving a molar Ca/P ratio of 1.58. As shown in Fig. 1, the BCP fractions were composed of rounded particles having the expected sizes. At high magnification, the surface of BCP particles appeared well-sintered with some remaining microporosity and a rough surface (Fig. 1(b)). Two techniques, SEM and laser scattering, were used to determine the particle size distribution. As shown in Table 1, the three fractions of BCP micro particles had average particle size of 4.2, 62 and 102 μm using the SEM method and 6, 67 and 138 μm using the laser scattering measurement. Both methods provided with similar results for average particle sizes. The particle size distribution of the 3 fractions is shown in Fig. 2. The 3 fractions exhibited a Gaussian particle size distribution centred approximately on their average value. Although some larger particles were present, most of the particles had size within the intervals <20 μm, 40–80 μm and 80–200 μm.

3.2 Histological analysis

The 3 fractions of BCP micro particles <20 μm, 40–80 μm and 80–200 μm were bilaterally implanted in quadriceps muscles of rats for 7, 14 and 21 days. The study was designed using a number of 8 implants per group. All animals (36 rats) recovered well from surgery without complications and all implantation sites healed. Histological observations indicated a normal fibrous tissue encapsulation of the BCP microparticles implanted in muscles. As shown in Fig. 3, several inflammatory cells were observed in the implantation area. These cells were multinuclear macrophages, giant cells and mature lymphocytes. The macrophage cells were principally in contact with the BCP microparticles while other cell types were present in the interstitial tissue between the BCP micro particles. Table 2 gathers the total number of cells and the thickness of the fibrous tissue capsule measured around the BCP micro particles. For the fraction < 20 μm, the total number of cells remained constant over the implantation periods of 7, 14 and 21 days. For micro particles in the range 40–80 μm, the total number of cells was approximately double after 7 days as compared to longer implantation delays of 14 and 21 days. Regarding the large micro particles of 80–200 μm, a slightly higher number of cells were found after 7 days than after 14 and 21 days. The comparison of

Table 1 Physicochemical properties of the BCP micro particles

Fractions	HA (wt%)	ß-TCP (wt%)	Ca/P	CO_3	CaO	Mean particle size ± S.D. (μm)	
<20 μm						$4.2 \pm 2.75^{(a)}$	$6.1 \pm 6.1^{(b)}$
40–80 μm	50.2	49.8	1.58	No IR peaks	Not detect.	$61.8 \pm 33.2^{(a)}$	$66.9 \pm 11^{(b)}$
80–200 μm						$102.1 \pm 60.2^{(a)}$	$137.6 \pm 26.8^{(b)}$

[(a)]Measured by BSEM and image analysis,
[(b)]Measured by laser scattering.

Table 2 Total average number of cells and thickness of the fibrous tissue capsule measured for BCP micro particles with size of $<20\ \mu$m, 40–80 μm and 80–200 μm implanted in muscles of rats for 7, 14 and 21 days ($*N = 8$)

Fractions	Implantations time (days)	Total average number of cells per field $\times 10$	Fibrosis*
$<20\ \mu$m	7	860 ± 285	1.7 ± 0.6
	14	926 ± 341	1 ± 1.1
	21	947 ± 238	1.5 ± 0.6
40–80 μm	7	755 ± 167	1 ± 0.8
	14	480 ± 23	1.2 ± 0.9
	21	460 ± 36	0.5 ± 0.5
80–200 μm	7	678 ± 134	1.25 ± 0.5
	14	550 ± 73	1 ± 0.8
	21	517 ± 98	2

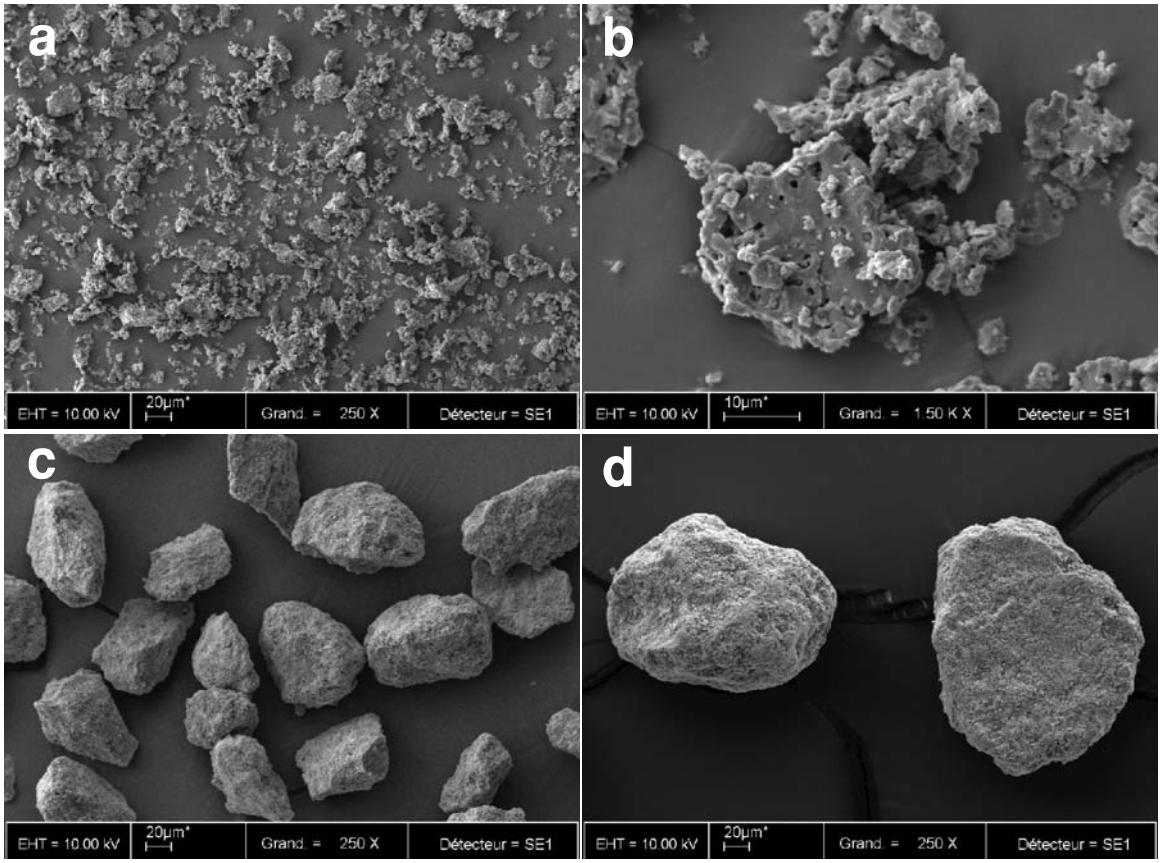

Fig. 1 SEM micro graphs of the BCP micro particles. (a, b) particle size $<20\ \mu$m, (c) 40–80 μm and (d) 80–200 μm. (b) Note the surface structure of micro particles

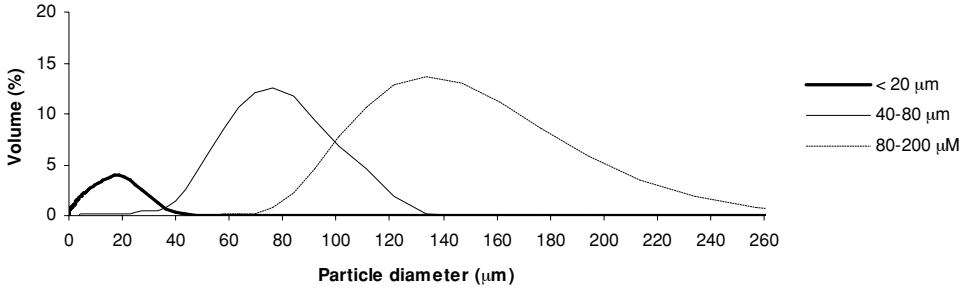

Fig. 2 Microparticles size distribution of the 3 BCP fractions measured by laser scattering

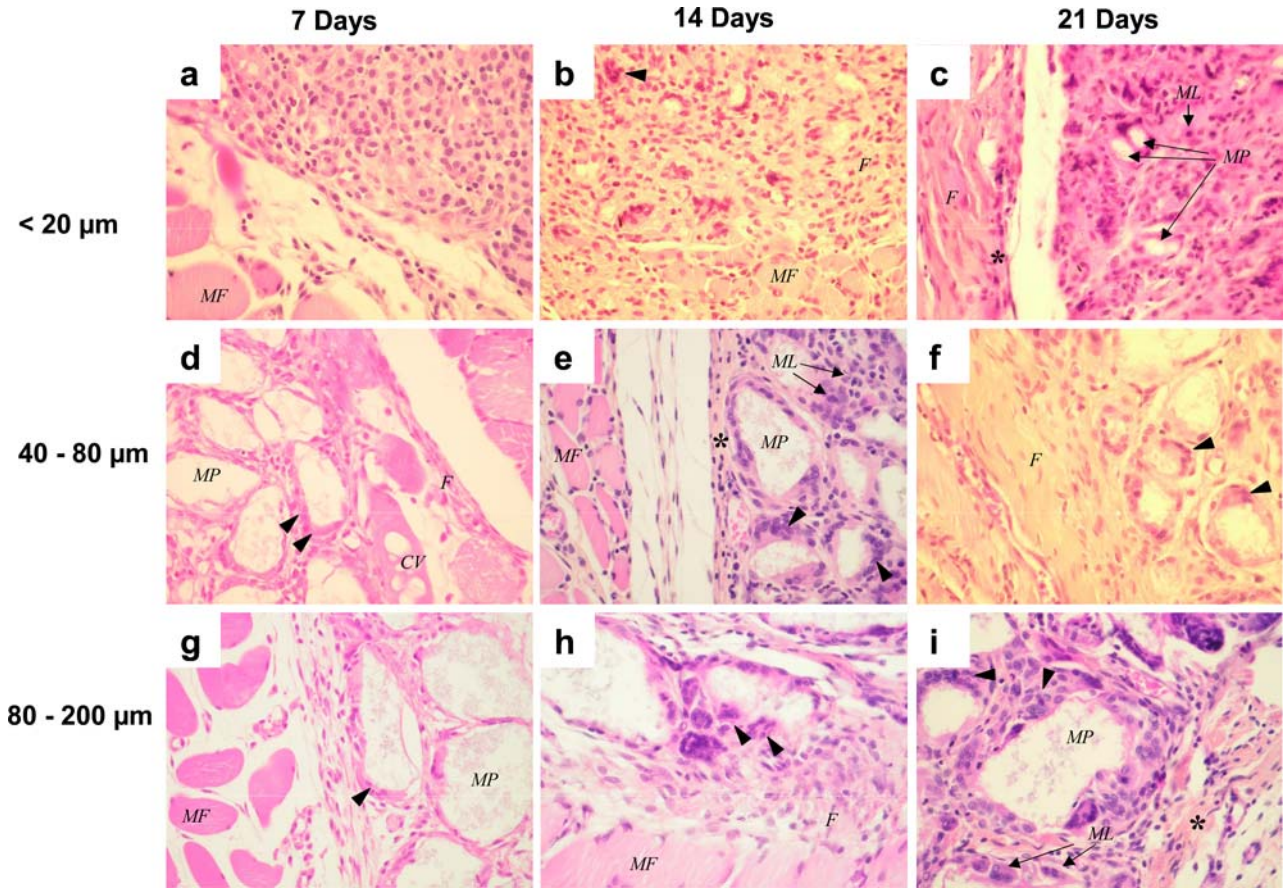

Fig. 3 Histological sections of BCP microparticles implanted in rats muscles. MF: muscle fiber, *: conjunctive capsule consists of fine collagen fibre, ML: mature lymphocyte, F: fibrosis which represents the last step of inflammation and isolate ceramic particles from rat muscles, MC: macrophage cells, MP: micro particles, CV: congestive blood vessels, Arrowheads indicated multinucleated giant cells. Magnification × 800

the cellular response given for the 3 fractions indicated that the total number of cells were significantly higher for BCP <20 μm than for 40–80 and 80–200 μm ($p < 0.0001$). A similar trend was observed for the thickness of the tissue capsule around the implanted BCP micro particles. Fibrosis was similar for the smallest micro particles <20 μm over the 3 delays of implantation. For the intermediate size of 40–80 μm, fibrosis decreased with the implantation time while it maintained for the largest particles. However, the comparison of fibrosis for the 3 fractions was not significantly different due to the semi-quantitive scoring of the method.

Figure 3 illustrates the muscle tissue response towards the BCP micro particles of different size implanted for 7, 14 and 21 days. The muscles fibres (MF) are clearly identified around the implanted micro particles (MP). The BCP micro particles are not observable on these decalcified histological sections but their locations are visible as translucent spaces. For the smallest micro particles of <20 μm, numerous macrophages and some mature lymphocytes and giant cells were observed after all implantation delays (Fig. 3(a–c)). Non nucleated macrophages were noticed after 7 days (Fig. 3(a)). Various inflammatory cells and bounded macrophages were found after 14 days (Fig. 3(b)). At 21 days, a fibrous capsule surrounded the BCP microparticles <20 μm and the collagen layer was infiltrated by fibroblasts (Fig. 3(c)). For the intermediate fraction of 40–80 μm, a thin fibrous capsule (F) was formed after 7 days of implantation. Rather important vessel congestion was also observed (Fig. 3(d)). Vessel congestion is a fundamental step in body inflammatory reaction and characterized by an increase of blood vessel diameter.

A fibrous capsule was present on sample sections after 14 and 21 days (Fig. 3(e, f)). As previously observed, macrophages and Giant cells were the predominant type of cells while only few mature lymphocytes were observed in between the BCP micro particles of 40–80 μm. The cellular and tissue response towards BCP microparticles of 80–200 μm is shown in Fig. 3(g–i)). Neither inflammation nor fibrosis was observed after 7 days around the large implanted micro particles (Fig. 3(g). After 14 days, a fibrosis capsule was formed with a few cell reactions compared to 21 days (Fig. 3(h) and (i)). A congestive capsule with abundant lymphocytes and macrophages around BCP 80–200

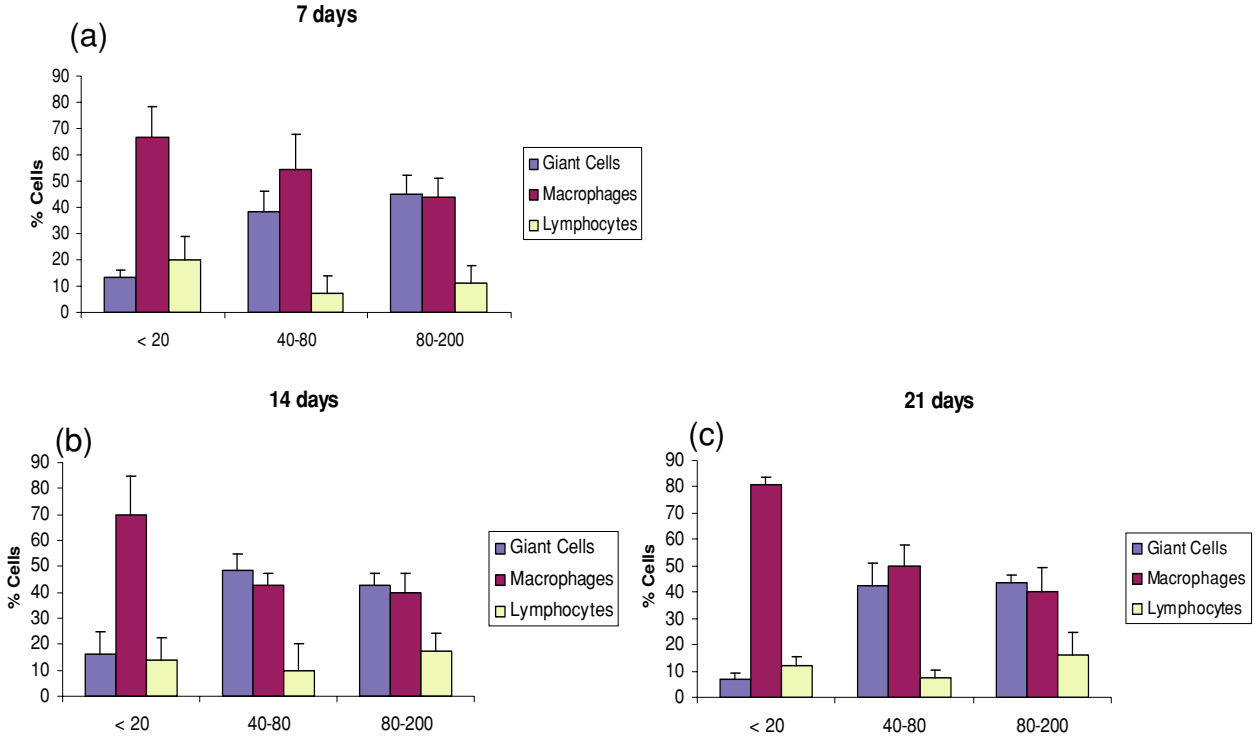

Fig. 4 Percentages of giant cells, macrophages and lymphocytes counted on histological sections of BCP micro particles with size of $<20\,\mu$m, 40–80 μm and 80–200 μm implanted in muscles of rats for (a) 7 days, (b) 14 days and (c) 21 days

μm at 21 days (Fig. 3(i)) was more distinct. Qualitatively, the comparison of the population of lymphocytes for the 3 fractions suggested that less lymphocytes were present within implanted 40–80 μm than in <20 and 80–200 μm BCP microparticles. The relative percentages of macrophages, giant cells and lymphocytes were plotted as function of implantation time and size of BCP micro particles (Fig. 4). The number of macrophages was relatively higher for the smallest fraction of <20 μm than for the intermediate 40–80 μm and largest 80–200 μm ones for all implantation time ($p < 0.0001$). The number of macrophages maintained over time for all considered groups of BCP particles. Whatever the delay of implantation, the giant cells were more important in the group of intermediate and largest size of particles than in the smallest size of BCP micro particles ($p < 0.0001$). The relative percentage of giant cells was comparable for the intermediate and largest size of particles and not significantly different after 7, 14 or 21 days. The number of lymphocytes was comparable for the 3 fractions and after the 3 delays.

4 Discussion

This study demonstrated that BCP micro particles with an average size around 5 μm (Table 1) induced an inflammatory response with macrophages and giant cells while implanted in muscles of rats. The cellular and tissue response towards larger BCP particles was relatively moderate. The role of particle size of CaP ceramics towards inflammation and osteogenesis has been poorly investigated *in vivo*. Malard and coll. have shown that 10–20 μm implanted in femoral or tibial defects of rats provided with an early and strong inflammatory reaction. The larger particles with size ranging 80–100 or 200–400 μm induced a moderate inflammation. Additionally, the smallest size of BCP particles led to the best bone ingrowth together with the higher resorption/degradation rate. The same authors have found that significant higher number of multinucleated giant cells with bone marrow cells cultured in contact with 10–20 μm BCP micro particles than for large size. Other studies have shown that high amounts of TNF-α and IL-6 cytokines were produced by macrophages cultured in the presence of micro particles [17–23]. It has been observed that conditioned culture medium of macrophages exposed to micro particles stimulated osteoblasts to produce inflammatory mediators which play an important role in bone resorption [20, 22, 25]. It has been also observed that macrophages, osteoclasts, osteoblasts and stem cells are present at bone fracture sites and all these cell types contribute to the healing process simultaneously [26–28].

Several studies have recently reported that specific materials are able to induce bone formation after implantation for 6–12 weeks in ectopic sites [8–11]. For instance, bone induction has been observed with synthetic hydroxyapatite

HA ceramics, biphasic calcium phosphate BCP, tricalcium phosphate ceramics, calcium phosphate cements, bioglass and even with titanium oxide and alumina ceramics implanted into the muscles of rabbits, dogs, goats, baboons and pigs [11, 12, 30–34]. Osteoinduction by biomaterials is a complex biological phenomenon that is not yet fully understood. It has been shown that ectopic bone formation is both material and animal dependant. Furthermore, surface microstructure is thought to play a key role in the osteoinductive capacity of bioceramics [12]. The micro porosity is due to the uncompleted sintering of ceramics at relatively low temperature. Two possible mechanisms have been proposed to explain this intriguing phenomenon of bone formation associated with materials. Both mechanisms are associated with the ceramic microstructure and the two cascades of biological events may be occurring in the same time.

In the first hypothesis, the surface microstructure may promote both the precipitation of biological apatite and cell adhesion on the surface of the materials. It has been shown that micro pores below 5 μm in diameter greatly enhanced ceramic dissolution in body fluids and the reprecipitation of biological apatite at the surface [35]. Furthermore, the large surface area of microporous ceramics may favour the adsorption and entrapment of endogenous proteins including BMPs, which have a high affinity for calcium phosphates [36]. These osteogenic proteins on the surface of the material could promote both the adhesion and differentiation of progenitor cells into osteoblasts [37].

The second possible mechanism of biomaterial osteoinduction might be related to the release of microparticles due to the preferable dissolution of grain boundaries in body fluids. This release of micro particles might provoke an inflammatory reaction [14, 17]. The local release of inflammatory cytokines then stimulates circulating stem cells to differentiate into osteoblastic cells, thus producing bone tissue. This process could be similar to that observed during the healing of bone fractures by intra membranous ossification, where bone fragments are usually present. The degradation of the blood clot within a bone fracture might provoke a local inflammatory reaction leading to the release of cytokines. Those cytokines might in turn promote the differentiation of circulating stem cells into osteoblasts.

5 Conclusion

In this study, 3 sizes of BCP micro particles were characterized and implanted in muscles of rats for 7, 14 and 21 days. A similar fibrous tissue encapsulation was observed for the 3 groups while the total number of cells was significantly higher for BCP <20 μm than for 40–80 and 80–200 μm micro particles. Macrophages were relatively more present in the interstitial tissue for the smallest micro particles than for the intermediate and largest fractions. On the opposite, more giant cells were found in the presence of intermediate and largest size of particles than for the smallest. Lymphocytes were present in comparable numbers for the 3 fractions and at the 3 delays. Therefore, the BCP micro particles <20 μm initiated a superior inflammatory reaction than larger sizes. These micro particles with a mean diameter around 5 μm might mimic the release of grains associated to micro porous ceramics which have been found to be osteoinductive in various animal models.

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