Osteogenic responses to extraskeletally implanted synthetic porous calcium phosphate ceramics: an early stage histomorphological study in dogs

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In this experiment, synthetic porous calcium phosphate ceramics (hydroxyapatite–tricalcium phosphate) were prepared and implanted in dorsal muscles of dogs. The purpose was to study the biological processes prior to and during the morphogenesis of bone in extraskeletally implanted porous calcium phosphate ceramics. Specimens were harvested after implantation for 7, 15, 30, 45, 60, 90 and 120 days. Decalcified and undecalcified sections were prepared for alkaline phosphatase (ALP) histochemical localization and comparative histological analysis. The results show that bone morphogenesis in the pore regions of the extraskeletally implanted ceramics follows a complex process involving clot formation, vascular invasion, granulation-like tissue formation, polymorphic cell aggregation, osteoblast differentiation and bone formation. The characteristic feature preceding bone formation was polymorphic cell aggregation on the pore inner surface and near the invading capillaries or small venules. These cells were of various sizes and shapes, and some of them were positive for ALP activity. ALP-positive cell aggregates were more numerous where capillaries or venules were close to the pore inner surface. Osteoblast differentiation occurred within the cell clusters aggregated on the pore inner surface and bone matrix was secreted in direct contact with the ceramics. During bone formation, capillaries or small venules were always found close to the developing fronts of the osseous nidi. It is suggested that those cells which first appeared near the invading vasculature, the cells which aggregated on the pore inner surface and those cells which finally differentiated into osteoblasts may be interrelated in some way.

1. Introduction

Calcium phosphate ceramics are widely used as biomaterials because of their good biocompatibility as well as osteointegrative properties [1–4]. When implanted in an osseous site, these materials can bind directly with bone without an intervening fibrous layer. Generally, bone formation at a material surface is thought to occur by osteoconduction from surrounding osseous tissues, where the material acts as a guidance surface for the elaboration of new bone during normal healing in the implantation bed [5]. In recent years, osteogenesis in extraskeletally implanted porous calcium phosphate ceramics has received considerable attention [6–10]. The mechanism by which the extraskeletally implanted porous calcium phosphate ceramics induce the morphorgensis of bone is at present unknown. Previous studies have shown that porous calcium phosphate ceramics are capable of inducing osteogenesis when implanted in non-bony sites, but this ability depends on both the species of animals and the types of ceramic which have different phase compositions and porous structures [11–16]. The aim of this experiment was to study the early stages of tissue formation prior to and during the morphogenesis of bone, which will contribute to understanding the biological mechanism of this heterotopic osteogenesis phenomenon.

2. Materials and methods

2.1. Implant materials

Starting apatite powders were prepared by the wet precipitation method. The porous green body foamed by the H_2O_2 foaming method was sintered at 1250 °C for 3 h. The biphasic ceramic consisted of 65% hydroxyapatite (HA) and 35% β-tricalcium phosphate (β-TCP) with 61% porosity and an average pore size of 402 μm. Scanning electron microscopy (SEM) observations show many interconnected micropores (2–5 μm) on the macropore (200–600 μm) walls. X-ray diffraction and SEM characterization of the porous calcium phosphate ceramics were described in previous study [11]. A total of 28 cylinders (diameter,

0957–4530

4 mm; length, 5 mm) of porous HA–TCP ceramics were prepared for implantation.

2.2. Surgical procedure

Four male mongrel dogs, all adult and healthy, were used as the animal model in this study. Animals were anaesthetized with an intra-abdominal injection of 2.5% sodium pentobarbital. Porous HA–TCP ceramics cylinders were implanted in the dorsal muscles of the dogs, all far from osseous tissues. Four cylinders were implanted for each implantation end point. Specimens were harvested after implantation for 7, 15, 30. 45, 60, 90 and 120 days and fixed in 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4; 4 °C) or in 10% buffered formalin.

2.3. Histological preparation

For decalcified histological sections, fixed specimens were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) in 0.1 M Tris-HCl buffer (pH 7.4;4 °C), embedded in paraffin wax, cut into sections of 6–8 μm and stained with haematoxylin and eoxin (H–E). For undecalcified sections, the fixed specimens were dehydrated in an ethanol series, embedded in methyl methacrylate, cut into sections of 10–20 μm and then stained with methylene blue and basic fuchsin. For alkaline phosphatase histochemical sections, the harvested specimens were fixed in 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4;4 °C) for 8 h, decalcified in 10% buffered EDTA (pH 7.4;4 °C), embedded in low-melting-point paraffin wax, cut into sections of 8 μm and stained by the modified Mayahara method [17, 18].

3. Results

After intramuscular implantation for 7 days, the pore regions of the porous calcium phosphate ceramics were mainly filled by clotted blood, consisting of numerous erythrocytes and some fibrous tissue (Fig. 1a). Around the implants, fibrous connective tissue capsules formed. The fibrous capsules were sandwiched between the normal surrounding muscular tissue and the implants. The fibres of the capsules were mostly arranged parallel to the outer surfaces of the implants. The pore regions of the ceramics were filled with granulation-like fibrous connective tissue after intramuscular implantation for 15 days. This loose fibrous connective tissue consisted of active fibroblasts, macrophages and newly formed blood vessels embedded in an open network of fibres (Fig. 1b). After implantation for 30 days, the fibrous connective tissue formed in the pore regions were remodelled into a denser type, and the fibres were mainly arranged parallel to the pore wall surface. Polymorphic cell aggregates could be noted mainly at the inner surface of the interconnected pores and in the vicinity of capillaries or small venules, especially at the sites where capillaries or small venules were close to the pore wall surface (Fig. 2a). These cells were of various size and shapes and some of them were positive for alkaline

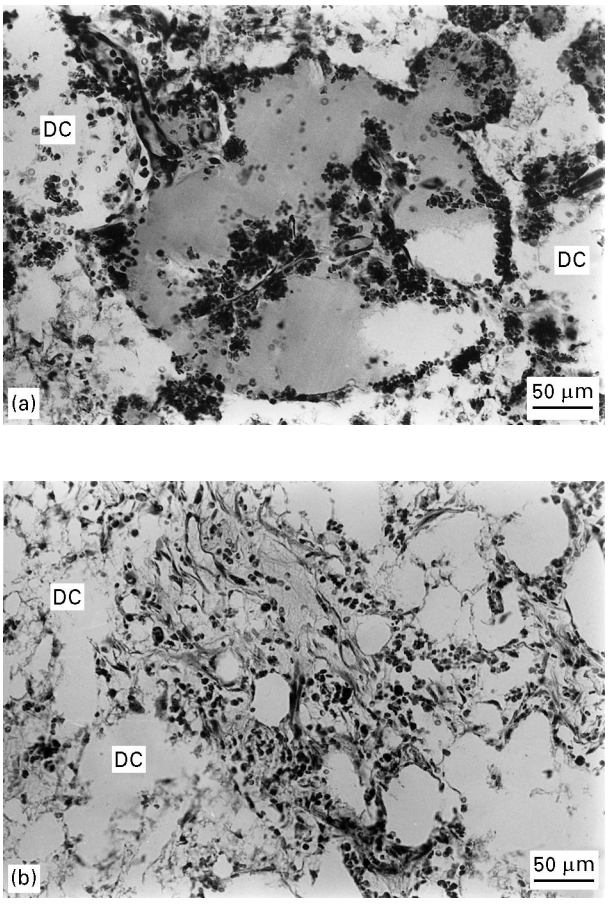
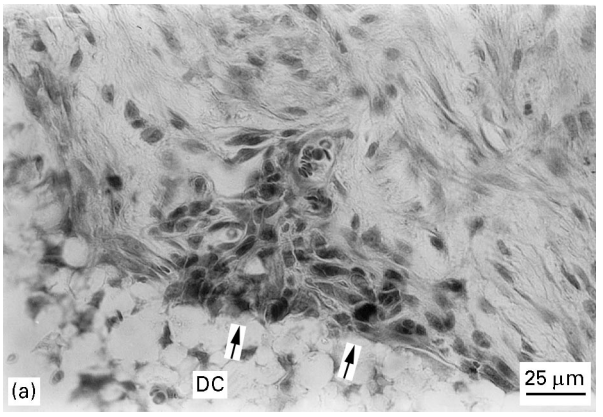
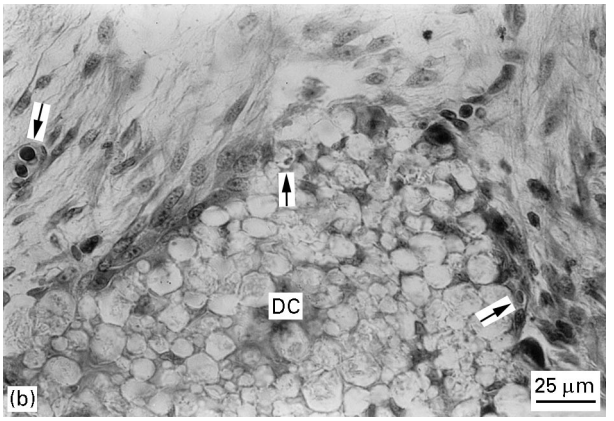


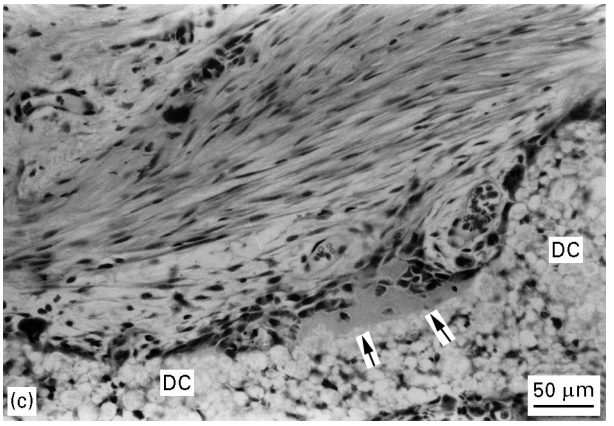
Figure 1 Decalcified sections of specimens harvested at day 7 and 15. (a) Blood clot formation in the pore regions of the ceramics at day 7. Numerous erythrocytes and few fibrous tissues could be noted. (b) Granulation-like fibrous connective tissue formation in the pore regions of the ceramics at day 15. DC, decalcified ceramics.

phosphatase (ALP) activity (Fig. 3a). Small numbers of multinucleated giant cells could be observed in direct appositon to the pore wall surface of the ceramics. At day 45, cell aggregation was more obvious and, in some cell aggregates, capillaries or small venules were likely to disappear (Fig. 2b). Osteoblast differentiation occurred directly within the polymorphic mesenchymal cell clusters aggregated at the pore inner surface. Osseous nidi lined by active osteoblasts could be noted in a few pores of some specimens harvested at day 45 (Fig. 2c and d). Bone formation occurred in direct contact with the ceramics without intervening fibrous layers. ALP staining was mainly localized in the cell clusters aggregated at the pore inner surface and in active osteoblasts which lined the developing fronts of the osseous nidi (Fig. 3b and c).

Obvious bone formation could be observed in the pore regions of all specimens harvested after intramuscular implantation for 60 days. The newly formed bone was mainly of the immature woven type and occurred in direct contact with the ceramics (Fig. 4a). The osteogenesis followed an intramembranous ossification without cartilage formation. During bone formation, capillaries and small venules were always found close to the developing fronts of the osseous nidi (Fig. 4b). At day 90 and 120, an extensive amount of bone, mainly trabeculated cancellous type, developed







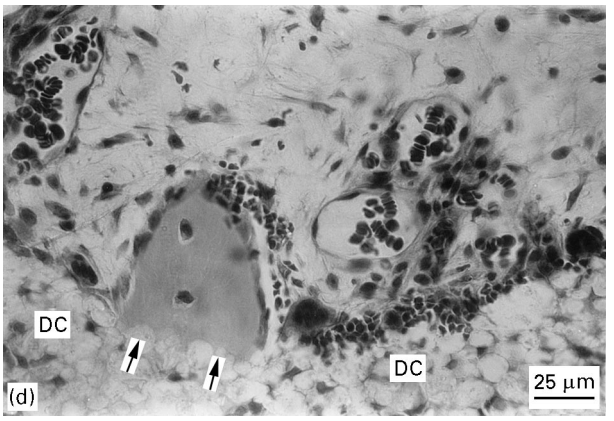
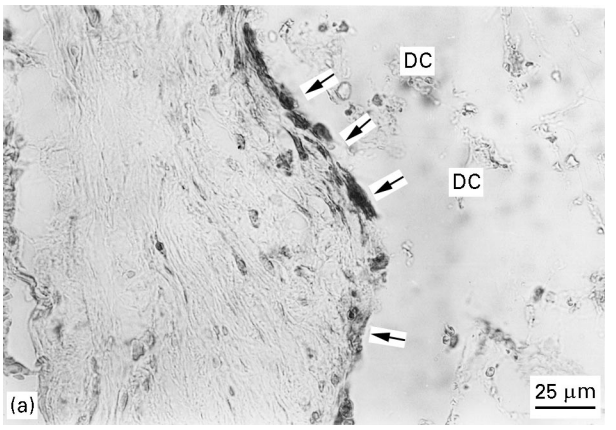
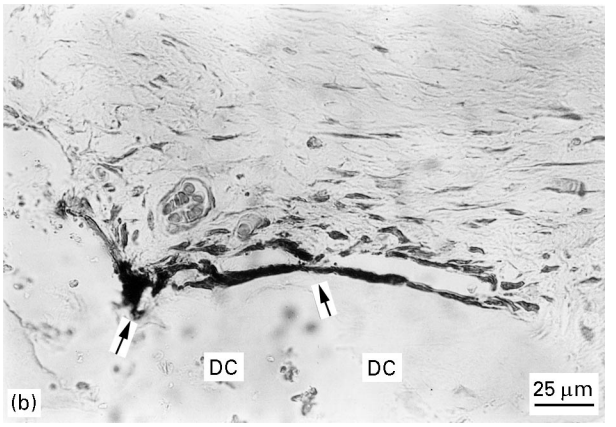


Figure 2 H–E-stained decalcified sections of specimens harvested at day 30 and 45. (a) A section of a specimen at day 30, showing polymorphic cell aggregation at the interface with the ceramics where capillaries are found close to the interface. (b) A section of a specimen at day 45, showing cell aggregation at the interface. Three very small capillaries (arrows) can be noted. (c), (d) Sections of a specimen at day 45, showing osseous nidi (arrows) formation in direct contact with ceramics. DC, decalcified ceramics.





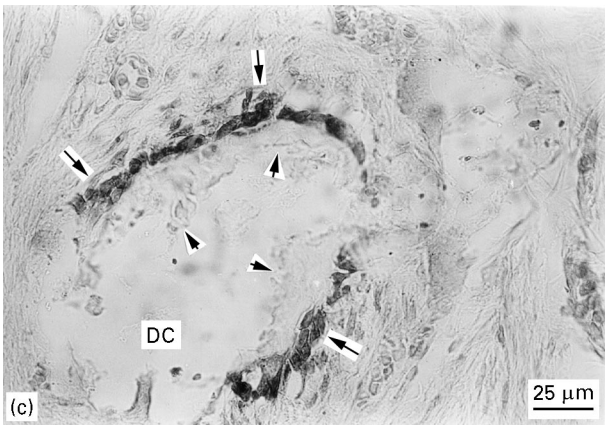
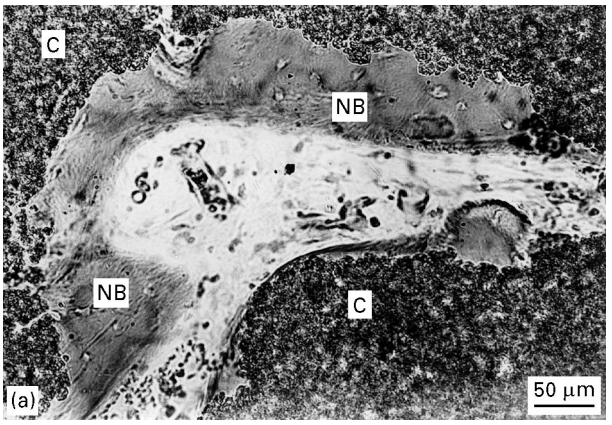


Figure 3 ALP histochemically stained decalcified sections of specimens harvested at day 30 and 45. (a) A section of a specimen at day 30, showing ALP-positive cells aggregation at the interface with the ceramics. (b) A section of a specimen at day 45, showing ALP-positive cell aggregation at the interface with the ceramics. (c) A section of a specimen at day 45, showing ALP-positive osteoblasts (arrows) lining the developing fronts of the osseous nidi (arrowheads). DC, decalcified ceramics.

in the pore regions of the ceramics (Fig. 5). The remainder of the void spaces were filled with marrow elements and fibrous connective tissue. In some specimens, remodelling of the newly formed bone occurred, but no mature lamellar bone and Haversian systems were observed at this time. Bone tissues mostly formed in the pore regions of the ceramics, and scarcely at the periphery of the implants. During bone formation, multinucleated giant cells could also be observed in direct apposition to the pore inner surface, but their number decreased with an increase in the bone



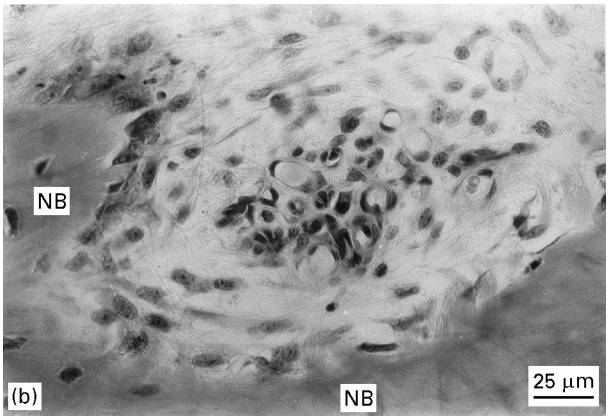
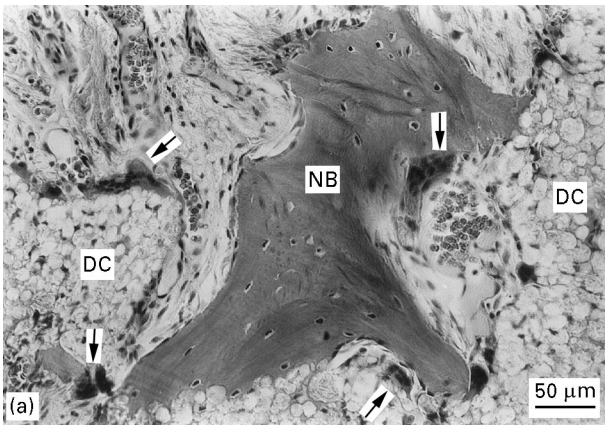


Figure 4 Decalcified and undecalcified sections of specimens harvested at day 60. (a) A undecalcified section, showing bone formation in direct contact with the ceramics in the pore region. (b) A decalcified section, showing the newly formed bone in the pore regions of the ceramics. Capillaries can be noted close to the developing fronts of the osseous nidus. C, ceramics; NB, newly formed bone.



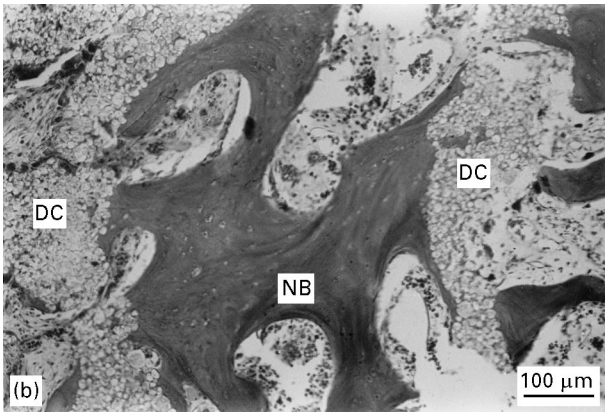


Figure 5 Decalcified sections of specimens harvested at day 90 and 120. (a) A section of a specimen at day 90, showing the newly formed bone in the pore regions of the ceramics. Multinucleated osteoclasts (arrows) were noted in direct apposition to the newly formed bone and to the pore wall surface of the ceramics. (b) A section of a specimen at day 120, showing extensive amount of bone formed in the pore regions of the ceramics. DC; decalcified ceramics; NB, newly formed bone.

bonding area of the pore inner surface. The shapes of these multinucleated giant cells were similar to those of osteoclasts that were remodelling the newly formed bone tissue (Fig. 5a). Fibrous capsules encapsulating the implants still existed after implantation for 120 days but became thinner and less dense in cellular density than at days 7 and 15.

4. Discussion

The mechanism by which the extraskeletally implanted porous calcium phosphate ceramics induce formation of bone in non-bony sites is at present unclear. Researchers would like to know how bone cells are differentiated in this non-bony environment, and which environmental factors play a key role in controlling osteoblast differentiation.

The results of this experiment have shown that tissue formation in the pore regions of the extraskeletally implanted ceramics followed a complex process involving first blood clot formation, secondly vascular invasion and granulation tissue formation, thirdly polymorphic cell aggregation and fourthly osteoblast differentiation and bone formation. The characteristic feature preceding bone formation was polymorphic cell aggregation at the pore inner surface and near the invading capillaries or small venules. These cells were of various sizes and shapes, and some of them were positive for ALP activity. ALP-positive cell aggregates were more obvious where capillaries or venules were close to the pore wall surface. Osteoblast differentiation occurred within the cell clusters aggregated at the pore inner surface and secreted bone matrix in direct contact with the ceramics. During bone formation, capillaries or small venules were always found close to the developing fronts of the osseous nidi. It is suggested that those cells which firstly appeared near the invading vasculature, the cells which aggregated at the pore inner surface and the cells which finally differentiated into osteoblasts may be interrelated in some way. It is possible that these various cells originated from the proliferation, differentiation and migration of the perivascular pericytes and endothelial cells. Other studies support this point of view. Previous studies have indicated that the pericytes and endothelial cells of capillaries and microvessels may function as resting stem cells and seem to be sources of matrix-forming cells in repair systems. Some workers have suggested that the perivascular endothelial cells and pericytes may be progenitor cells to the osteoblasts in periosteal osteogenesis, bone fracture repair processes and in bone-morphogenetic-protein-induced bone differentiation

[19–21]. An *in vitro* study by Brighton *et al.* [22] demonstrated that the capillary or microvessel pericytes exhibit phenotypic expression *in vitro* that is similar to that of *in vitro* bone cells.

Bone formation in the pore regions of extraskeletally implanted porous calcium phosphate ceramics mostly occurred in direct contact with the pore wall and developed towards the pore centre but scarcely started from the pore centre. It seems likely that the components deposited in direct apposition to the pore walls at earlier stages play crucial role in controlling the morphogenesis of bone. In general, it is suggested that the extraskeletally implanted porous calcium phosphate ceramics may act as a scaffold for osteogenin adsorption and locally initiation of bone formation [7, 15]. Previous studies have shown that synthetic porous calcium phosphate ceramics are capable of inducing osteogenesis when implanted in non-bony sites, but this ability depends on both the species of animal and the type of ceramic. Bone formation was found in porous calcium phosphate ceramics extraskeletally implanted in dogs and pigs, but not in rabbits, rats or goats [11]. Bone formation was found in porous calcium phosphate ceramics with microporous pore inner walls, but not in porous ceramics without microporous structures on pore inner walls [12]. Also, bone formation in porous pure HA ceramics was slower than in porous biphasic (HA–α-TCP or HA–β-TCP) and triphasic (HA–α-TCP–β-TCP) ceramics [13]. It seems likely that more environmental factors than osteogenin adsorption contribute to the bone morphogenesis in extraskeletally implanted porous calcium phosphate ceramics. The important environmental factors which are probably involved in inducing bone formation are first the interconnected macroporous structure which facilitates the ingrowth of blood vessels and cells, secondly the microporous structure of the macropore walls which increases the adsorption areas and may provide a favourable surface for cellular adhesion and differentiation, thirdly biomolecules and crystallized apatite layers deposited on the pore surface in the earlier stages and fourthly locally increased calcium ion concentration resulting from the degradation and dissolution of the ceramic. Further investigations are required to understand the biological processes and the key important environmental factors that regulate bone morphogenesis in extraskeletally implanted porous calcium phosphate ceramics, which will contribute significantly to further understanding of the tissue–implant interactions and further designing of bioactive bone substitutes.

Acknowledgement

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