Interactions of human osteoprogenitors with porous ceramic following diffusion chamber implantation in a xenogeneic host

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Porous calcium phosphate ceramics are useful bone graft substitutes on account of their osteoconductive properties and lack of toxicity, but they lack osteogenicity and are brittle in nature. Osteogenic properties, and increased biomechanical properties, could be induced by combining them with human bone-forming cell populations. Progress has been hampered both by the lack of a suitable experimental assay of *in vivo* human bone formation and a suitable *in vivo* test system with which to study such cells in association with biomaterials. Here, trabecular bone-derived cells and marrow stromal fibroblastic cells from four human donors aged between 14 and 27 y have been cultured *in vitro* then combined with a porous ceramic within diffusion chambers and implanted into athymic mice. Bone and cartilage formation was found within the chambers primed with cells cultured in the continuous presence of dexamethasone and ascorbate. These tissues were found in close apposition to the ceramic, confirming that the material is biocompatible and bioactive. These findings demonstrate both that appropriately primed human-cell populations can express the fully differentiated osteoblastic phenotype in the diffusion-chamber model, and also that this is a useful system in which to test the interactions of such cell populations with putative biomaterials.

1. Introduction

Porous calcium phosphate ceramics have been widely studied as bone-graft substitute materials, because they exhibit a lack of toxicity and are osteoconductive and potentially resorbable [1–3]. Bone bonds chemically to these ceramics and thus they are defined as being bioactive [4]. These materials alone are not normally considered to be osteoinductive or osteogenic, but recent reports indicate that bone induction may occur in dog muscle and skin following prolonged implantation [5, 6]. Nevertheless, bone ingrowth is usually observed only adjacent to pre-existing host bone, indicating that such materials alone would be inadequate for the repair of large bone defects [7–10]. Furthermore, the brittle nature of these ceramics precludes their sole use as load-bearing implants and thus they are mainly applied in granular form as bone fillers or as plasma-sprayed coatings on to implant materials [11].

Stratagems to induce osteogenic properties and potentially improve the biomechanical aspects of porous resorbable biomaterials are the incorporation of osteoinductive agents or osteogenic cells. Bone-inductive agents have been used experimentally in combination with a number of biomaterials with some success [12, 13]. Bone itself has a notable potential for complete tissue regeneration following injury [14]. This extraordinary regenerative capacity could be put to use in the repair of skeletal defects by impregnating porous materials with osteogenic cell populations derived from bone [15, 16]. Evidence suggests that bone regeneration occurs via activation of specific stem cells residing in close proximity to bone surfaces [17–19]. A variety of methods for *in vitro* cell culture of these osteogenic populations from experimental animals and the human is now available using bone marrow-derived [17], bone fragment-derived [20–22] and periosteum-derived [23, 24] cells.

Experiments studying the repair of rat femoral diaphyseal defects show that impregnation of porous ceramic with syngeneic bone marrow cells to form a hybrid material promotes enhanced osteogenesis within the ceramic and greater union to the host bone [9]. When such hybrid material is implanted subcutaneously or intramuscularly in rats, bone formation is observed within the pores in direct contact with the ceramic. There is a concomitant increase in compressive strength and rigidity of the material [10]. Furthermore, culture-expanded populations of rat marrow cells form bone more quickly than fresh

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marrow cells, suggesting potential clinical use of such amplification techniques [25].

When cultured cells combined with a ceramic are implanted into a xenogeneic host, such as is necessary in studies with human tissues, the findings are more complex. Using such a system, Goshima *et al.* [26] demonstrated a biphasic phenomenon of osteogenesis. The initial bone formed is of donor origin and this is subsequently remodelled and replaced by host-derived bone tissue. A similar situation occurs when cultured chick periosteal cells are implanted subcutaneously in athymic mice [27]. Cultured human periosteal cells have also been studied, but osteochondrogenesis was only observed with cells from donors of 19 y of age or less [24]. Again, host vasculature was observed in the pores 1 week after implantation, leading to uncertainty as to the species of origin of the bone tissue formed. Haynesworth *et al.* [16] demonstrated bone formation within porous ceramic containing culture-expanded adherent human marrow stromal cells implanted subcutaneously in athymic mice, and showed that the initial bone formed was of human origin by using a species-specific antibody. However, host vasculature again filled the pores 1 week after implantation, suggesting early interaction of implanted cells with host-derived cells.

An experimental approach to isolate the implanted cell population from invasion by host cells and vasculature is to use the closed environment of a diffusion chamber. This is an established *in vivo* experimental system to study directly potentials for cellular differentiation [28, 29]. These chambers are bounded by membranes permeable to tissue fluids but not to cells and may be implanted at appropriate sites into suitable hosts. Because host cells are excluded, tissue formed is assured to be of donor origin [30]. The osteogenic capacities of freshly isolated bone marrow cell suspensions and cultured marrow cells from a variety of experimental animal species, have been documented extensively by using the diffusion-chamber system [17, 31–33].

Studies using human osteogenic cell lines within diffusion chambers have been less successful. Human trabecular bone-derived cells and marrow stromal cells have been shown previously not to express osteogenic potential in the diffusion chamber [16, 34, 35]. Uchida *et al.* [36] produced some cartilage and a very little mineralized material with cultured periosteal cells from children aged 8–12 y, and Nakahara *et al.* [24] found that enzymatically released human periosteal cells produced only cartilage in diffusion chambers with low frequency, and then only with donors of less than 7 y of age. Gotoh *et al.* [37] reported bone formation in diffusion chambers by cells from a 7 y old male but disorganized mineral deposits within the chamber appear pathological. Bab *et al.* [30] reported that when fresh human marrow cell suspensions were implanted, donors of 4 and 8 y of age yielded minor deposits of von Kóssa positive material in two out of four diffusion chambers; marrow from all adult donors formed solely unmineralized fibrous tissue.

An advance in the use of this experimental system has been made by our recent demonstration that human bone tissue is formed consistently within diffusion chambers by human bone-derived and marrow stromal fibroblastic cells when cultured under appropriate conditions [38]. This has enabled the application of the diffusion chamber system to determine the direct interactions of biomaterials with human bone-forming cells. In the present studies, we have investigated the interactions of cultured human bone-derived and marrow stromal fibroblasts, and fresh bone marrow suspensions, with a hydroxyapatite/tricalcium phosphate ceramic in this experimental system.

2. Materials and methods

Trabecular bone explants from the posterior iliac crest of four patients (14–27 y old) undergoing spinal surgery were used to prepare cultures of marrow stromal cells and trabecular bone-derived cells. Only bone which would otherwise have been discarded was used with the approval of the Hospital Medical Staff Committee. Marrow stromal and bone-derived cells were prepared as described [38]. Briefly, trabecular bone explants were washed thoroughly in culture medium and the marrow cells recovered from the washings by centrifugation. Eight pieces (approximately $2\,mm \times 2\,mm \times 2\,mm$) of trabecular bone were cultured in each $80\,cm^2$ culture flask (Nunclon, Denmark), and for the marrow cultures, 1.3×10^8 total cells were inoculated into each $80\,cm^2$ culture flask. Both populations were cultured in Dulbecco's Modification of Eagle's Minimum Essential Medium (DMEM; 10 ml per flask) containing 10% (vol/vol) of heat-inactivated foetal calf serum (FCS, Globepharm Ltd, Esher, Surrey, UK) and 100 μM 1-ascorbic acid 2-phosphate (Takeda Chemical Industries, Tokyo, Japan). After 5 d the non-adherent cells were removed from the marrow cultures by rinsing with phosphate-buffered saline minus calcium and magnesium (PBS^-), and in both cell systems complete media changes were performed at 5 d then twice-weekly thereafter. From explanation, one-half of the cultures were supplemented with 10 nM dexamethasone and one-half were not supplemented.

Cells were recovered by rinsing the cell layers twice with serum-free DMEM and incubation for 2 h in DMEM containing 25 U/ml highly purified collagenase (Clostridium Histolyticum, type VII; Sigma Chemical Company, Poole, UK) and 4.8 mM total calcium. The cells were washed twice in PBS^- and incubated for 5 min in trypsin/EDTA solution (0.05% trypsin and 0.02% ethylenediaminetetraacetic acid in PBS^-, pH 7.4) at 37 °C until the cell layer separated. A single cell suspension was prepared by repeated gentle dispersion through a 19 gauge hypodermic needle and filtration through a cell strainer (70 μm pore size, Falcon, Becton Dickinson Labware, N.J.). Each cell type was resuspended in DMEM at 10^7 cells ml^{-1}.

The diffusion chambers were constructed with Millipore membranes of pore size 0.45 μm (Millipore UK Ltd, Harrow, UK) and contained discs of a porous

ceramic (60% hydroxyapatite/40% tricalcium phosphate with a mean pore size of 400 μm, Zimmer Corporation, Warsaw, IN). The cell suspension (100 μl; 10^6 cells) was applied to a partially constructed diffusion chamber with a single membrane attached containing the ceramic, and the other membrane was then carefully glued into place.

The chambers were implanted intraperitoneally into athymic mice (MF1 nu/nu; 1 month old; 18 g; Harlan UK Ltd, Bicester, UK). Fresh marrow cell suspensions from two donors were also inoculated separately into diffusion chambers containing discs of ceramic, with 1.5×10^7 total cells per chamber. In addition to primary cultures from all the donors, third passage bone-derived and marrow stromal cells from one donor (14 y old) were also cultured and implanted in diffusion chambers with ceramic.

After 11 weeks, the mice were sacrificed and the chambers removed and fixed overnight in 95% ethanol at 4 °C. No chambers showed evidence of host vascular penetration. The specimens were embedded undecalcified in hydroxymethylmethacrylate resin, sectioned at 5 μm, and stained by toluidine blue, the von Kóssa method, and for alkaline phosphatase expression [32].

3. Results

With all four donors, both bone-derived (Fig. 1a) and marrow stromal cells (not shown) cultured in the absence of dexamethasone and implanted at first passage into diffusion chambers for 11 weeks, generally showed fibrous tissue only in relation to the ceramic. The cells appeared healthy and in close proximity to the ceramic, demonstrating the lack of toxicity of this

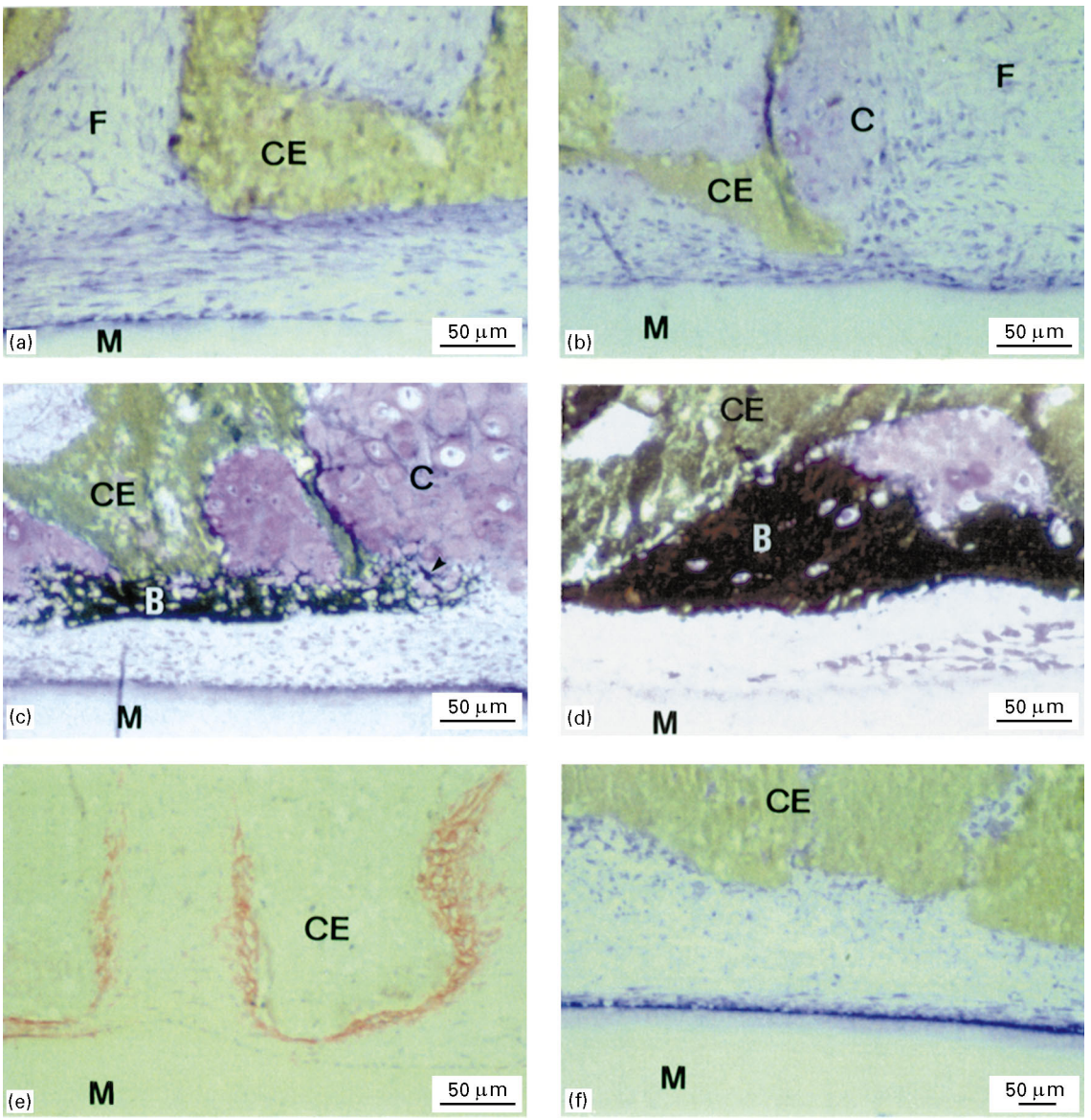

Figure 1 Histological sections of diffusion chambers containing discs of ceramic after implantation with the following cell populations: (a) bone-derived cells cultured in the absence of dexamethasone showing fibrous tissue only; Toluidine blue/von Kóssa; (b) Bone-derived cells cultured in the absence of dexamethasone showing an area of cartilage differentiation; Toluidine blue/von Kóssa; (c) Marrow stromal cells cultured in the presence of dexamethasone showing bone and cartilage formation; Toluidine blue/von Kóssa; (d) Marrow stromal cells cultured in the presence of dexamethasone showing integration at the interface of the ceramic and the osteogenic tissue; Toluidine blue/von Kóssa; (e) Bone-derived cells cultured in the presence of dexamethasone showing positive staining for alkaline phosphatase (red) in the membranes of cells adjacent to the ceramic; Alkaline phosphatase/haematoxylin; (f) Fresh marrow suspension showing little evidence of cell proliferation; Toluidine blue/von Kóssa. (M = Millipore membrane, CE = Ceramic, F = Fibrous tissue, C = Cartilage, B = Bone, Arrow = Area of cartilage mineralisation)

material. In one chamber of bone-derived cells from a 27 y old donor there were small areas of cartilage differentiation, but this was unmineralized and no bone tissue was seen (Fig. 1b). This cellular differentiation was observed adjacent to ceramic, with the remainder of the chamber being filled with fibrous tissue. These chambers were largely alkaline phosphatase negative, although small alkaline phosphatase positive areas were seen in some chambers (not shown).

With all four donors, both bone-derived (not shown) and marrow stromal cells (Fig. 1c, d) cultured in the continuous presence of dexamethasone and implanted at first passage into diffusion chambers for 11 weeks, showed bone and cartilage formation. The bone tissue formed comprised rows of active osteoblasts, seams of osteoid, and mineralized bone with osteocytes in lacunae. Deeper into the chambers, cartilage formation was observed, and there were areas of cartilage mineralization (Fig. 1c). Intense alkaline phosphatase activity was observed in the areas of bone formation (Fig. 1e). The cartilage and bone both formed an integrated interface with the ceramic (Fig. 1d).

No significant differences were observed between the bone-derived and marrow stromal cell populations in terms of the amounts and types of tissue formed. Findings in the chambers containing third passage cells from the one donor studied were similar to those containing cells at first passage (not shown).

No bone or cartilage formation was seen in any of the chambers containing ceramic which had been filled with fresh marrow suspensions from two donors (Fig. 1f). There was little evidence of cellular proliferation in these chambers, save for some fibrous tissue a few cells thick close to the Millipore membrane.

4. Discussion

These studies show human bone formation in diffusion chambers in close association with ceramic using appropriately cultured human trabecular bone-derived cells and marrow stromal cells. The results demonstrate that the diffusion chamber is a useful experimental system to study the compatibility of biomaterials with human bone development. Although not ideal because of the lack of bone tissue remodelling due to the restriction of blood vessel ingress, it affords an additional appraisal system for evaluation of human tissue integration with biomaterials such as the bioactive ceramic used here. As we and others have reported previously [16, 38], bone forms in association with this ceramic in subcutaneous implantations impregnated with cultured human bone-derived and marrow stromal cells. In all these studies no cartilage was observed and it is likely that the cartilage formation seen in diffusion chambers is due to a relatively poor oxygenation state deep within the chambers [39, 40].

We have shown previously that no bone tissue develops from fresh human marrow cell suspensions alone in diffusion chambers and this lack of potential is corroborated in the present study with the addition of ceramic. As noted earlier [38], the production of bone by these cultured cells suggests either that the numbers of osteoprogenitor cells are increased by *in vitro* culture, as signified by the work of a number of authors [25, 41, 42], or that a normal marrow inhibitor of osteoprogenitor proliferation is reduced. There appears to be significant differences between the potential for bone formation in diffusion chambers by fresh marrow from experimental animals [32] compared with our experience with the human. Whether this signifies lesser numbers or lower viability of stem cells implanted in diffusion chambers in the human preparations, or reflects some technical feature, is not known.

Again in agreement with our recent work [38] no apparent difference in developmental potential was seen between the marrow and bone-derived human cells in association with the ceramic. This is contrary to the findings in animal experiments that marrow cell populations appear to be enriched in early progenitors, whereas isolated, bone-derived osteoblast-like cell populations tend to have more restricted lineage potentials and lose their potential for osteogenesis after short periods of culture [43].

The results presented here, showing the close interface between the osteogenic tissues and the ceramic, confirm the bioactive nature of this material and demonstrate its biocompatibility with human skeletal tissues. We conclude that the osteogenic capacity of appropriately primed populations of human cells derived from bone and marrow can be used to assess the development of human bone in close association with a ceramic used in clinical practice. This indicates that this experimental procedure will be a useful method to assess the interactions of human bone-forming cells with new putative skeletal biomaterials. In addition, this system may have value in other human cell systems where knowledge of specific tissue cell interactions with biomaterials is of importance. Combinations of osteogenic cell populations which have been culture-expanded *in vitro*, together with a space-filling biomaterial carrier such as a porous ceramic, are likely to prove useful as composite bone-grafting materials, having both osteogenic and osteoconductive properties.

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