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Bone cell grafts in bioreactor: a study of feasibility of bone cell autograft in large defects

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Cancellous bone cells were isolated from adult dogs, introduced into cell culture, subcultured and grown on hydroxylapatite granules. Cells immobilized on these granules were used to make bioreactors which were implanted in dog ulna diaphyse to fill osseous defects. The bioreactor implantation constituted a bone cell autograft and showed bone formation in a reactor containing cultured cells but not in the control reactor containing hydroxylapatite granules without cells. These results indicate that hydroxylapatite material can be used in bioartificial organs. The properties of hydroxylapatite used in bone reconstruction are due to the cells and extra-cellular matrix immobilized on its surface.

1. Introduction

Stromal bone cells have been reported to play crucial roles in the furnishing of stem cells for osteogenic cell lineage. Studies on bone development, fracture healing and bone cell transplantation in diffusion chambers have suggested the presence of mesenchymal progenitor cells in the stromal tissue of cancellous bone. Friedenstein [1] has isolated from cancellous bone a self-perpetuating population of determined osteogenic precursors. In monolayer cultures, these cells form clones of fibroblast-like cells. Owen [2] has proposed a hypothesis for differentiation in the stromal system that is analogous to that in the haematopoietic system, where stromal stem cells give rise to committed progenitors for different cell lines (their number and hierarchy are not completly known). They include fibroblastic, osteoblastic, osteoclastic and possibly other lines.

It has been demonstrated that suspensions of single cells derived from cancellous bone tissue form a mixture of fibrous and chondro-osteogenic tissue when incubated within diffusion chambers implanted *in vivo* [3, 4]. Histomorphometric and biochemical studies showed that the mixture of bone, cartilage and fibrous tissues formed within the chambers was generated by small numbers of precursor cells with a high capacity for proliferation and differentiation, i.e. cells with stem characteristics.

The ability of cancellous bone cells grown from a single colony *in vitro* to form osteogenic tissue in diffusion chambers was confirmed by Friedenstein *et al.* [5]. Differences in proliferative activity, in synthesis of extracellular matrix components and in hormonal and biochemical responses by individual clones suggested that their origin is a heterogeneous population of precursors, some of which have multipotentiality [6, 7].

With the above studies as a base, we attempted to liberate the mesenchymal cells containing osteogenic lineage from bone trabecules to expand this population for a first time mitotically using cell culture. Then, we immobilized cells on porous hydroxylapatite (HAP) granules before growing this cell line on the substrate and expanded the population for a second time. The objective of such an undertaking was to increase the number of potentially reparative cells available for a cell graft in an osseous defect. To improve the graft efficiency (vascularization, mechanical properties) and the handling properties of the device, we made a bioreactor containing the cell line immobilized on HAP carriers. The reactor was implanted in an osseous defect on dog ulna. The work constituted a feasibility study of such bone cell autografts.

2. Materials and methods

2.1. Osteogenic cells

A cancellous bone biopsy was done on a dog metaphyseal humerus. The biopsy is cut into small cubes,

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transferred in a Petri dish and carefully washed with phosphate-buffered saline (PBS). Then, bone explants were transferred into DMEM medium supplemented in 10% foetal calf serum (IBF) and glutamine (5 mM), and incubated at 37 °C in a 5% CO_2 and 98% R.H. atmosphere for 3 weeks. After this incubation, bone explants were transferred to a collagenase (SIGMA) solution (2 mg/ml PBS) at 37 °C for 2 h with vortexing for 10 s every 30 min. The supernatant was centrifuged and resuspended in DMEM-supplemented foetal calf serum and glutamine and plated in Falcon dishes at 37 °C in 5% CO_2, 98% R.H. atmosphere until they reach confluence. They were then suspended in DMEM-supplemented foetal calf serum and glutamine with a trypsin–EDTA solution and seeded on HAP porous granule bed (4 g) at 1×10^8 cells g^{-1}. The culture was grown for 2 weeks at 37 °C in a 5% CO_2 and 98% R.H. atmosphere, and the culture medium was replaced every day.

2.2. Cell immobilization

A bed of porous granules (3 mm diameter) made of pure HAP was used as culture support. The average pore size was 90 μm. HAP granules (4 g) were deposited in a 6 cm diameter Petri dish and seeded with a suspension of bone cells (4×10^8 cells/4 ml). Bone cells were fixed on granules within 1 h. The culture medium was replaced each day and cells were grown on the mineral matrix for 4 weeks at 37 °C in a 5% CO_2 atmosphere with 98% R.H.

2.3. Bioreactor

A hollow cylinder made of polymethylmethacrylate (PMMA) with a diameter of 1 cm and a length of 2 cm was filled with HAP granules on which bone cell lines were immobilized. A control bioreactor was realized with the same envelope (PMMA) filled with HAP granules without any cells grown on it.

2.4. Implantation protocol

Diaphyseal ulnas of 7 dogs were osteomized on a length of 2 cm. The osseous defect was filled (Fig. 1) with an active bioreactor (containing stromal cell line on HAP granules) in the left upper limb and with the control (HAP granules without cells) bioreactor on the right upper limb of the same dog. The device was fixed by a screw plate bridging the defect. Each dog was implanted with its own cells (cell autograft) to avoid immune response against the cell line.

2.5. Control

The device (active and control bioreactors) was harvested at intervals of 3 days (1 dog), 3 weeks (2 dogs), 2 months (2 dogs) and 4 months (2 dogs); fixed with 10% formalin in PBS (pH 7.5) and embedded in PMMA. Sections were stained with Masson trichrome and by the Von Kossa method counterstained with Toluidine Blue [8]. X-radiograph controls were made at 2 week intervals and the dogs were injected weekly with tetracycline.

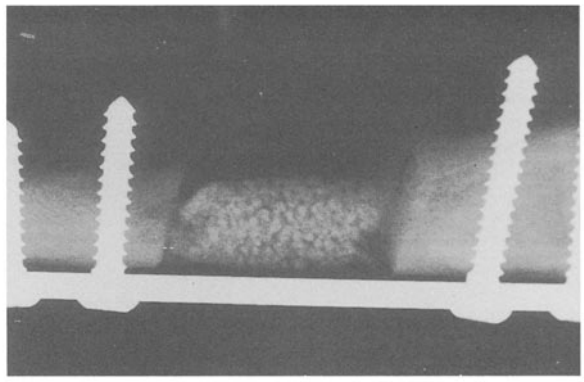

Figure 1 X-radiograph control of the active reactor after implantation.

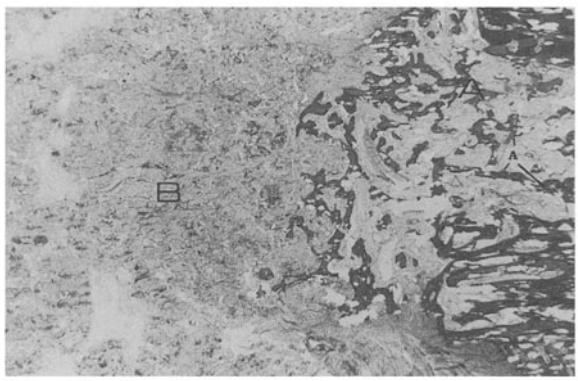

Figure 2 Osteogenesis in the active reactor 3 weeks after implantation. Trabecular bone (A) is located in a vascularized zone going from the implanted bone to the centre of the reactor containing undifferentiated tissue (B). Goldner trichrome, × 15.

3. Results

Cells immobilized on HAP were still alive 3 days after the implantation in the osseous defect. In the control reactor, HAP granules were embedded in a red cell and polynuclear exudate.

An osteogenesis was seen in the active reactor 3 weeks after the implantation, spreading from the junction area between bone and reactor to its centre (Fig. 2). This centre is occupied by undifferentiated cells and acellular connective tissue containing many reticular fibres. There was no cartilaginous tissue. At a higher magnification, osteoblasts appeared to synthesize an osteoid substance on and between HAP granules. Ossification occurred following an intramembranous ossification pattern and this process lead to cancellous bone formation. Some HAP granules were fragmented and some fragments were phagocytozed by macrophages and fibroblasts. Bone and conjunctive tissues were present in regions invaded by blood vessels. The diaphyseal bone in which the reactor was implanted was not dead and an osteoid substance could be seen at the interface between the bone and the exogenous material.

The control reactor was implanted in dead bone, osteoplasts were empty and there was bone resorption by osteoclasts at the interface (Fig. 3). The device was separated from bone by a dense connective tissue, and only a few undifferentiated cells were present in the

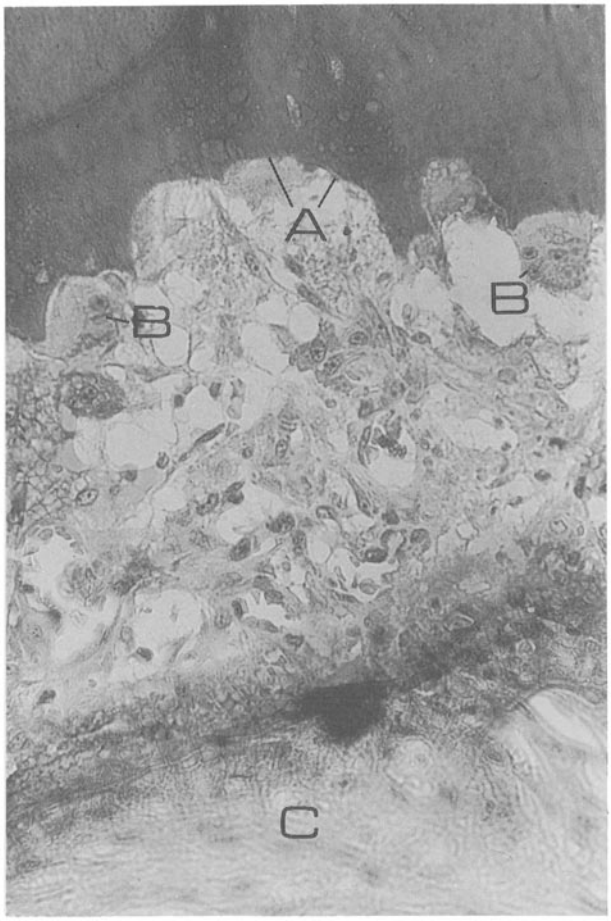

Figure 3 Bone resorption (A) at the bone–control reactor interface by osteoclasts (B) after 3 weeks. Bone is separated from the reactor by connective tissue (C). Goldner trichrome, × 400.

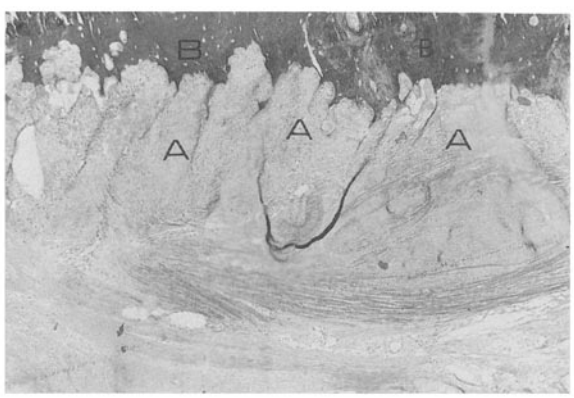

Figure 4 Connective tissue (A) separating the control reactor from the bone tissue (B) 3 weeks after implantation. Goldner trichrome, × 100.

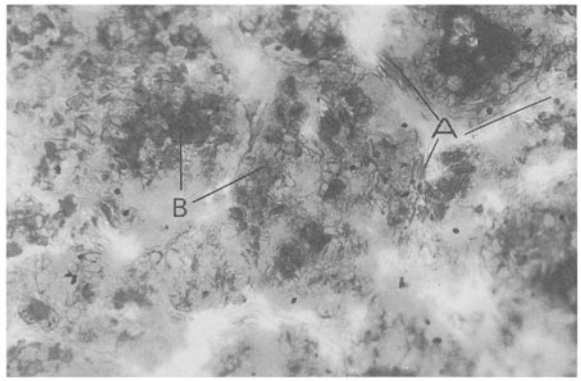

Figure 5 Poor cellular connective tissue (A) appearing in the control reactor around the HAP granules (B) 3 weeks after implantation. Masson trichrome, × 50.

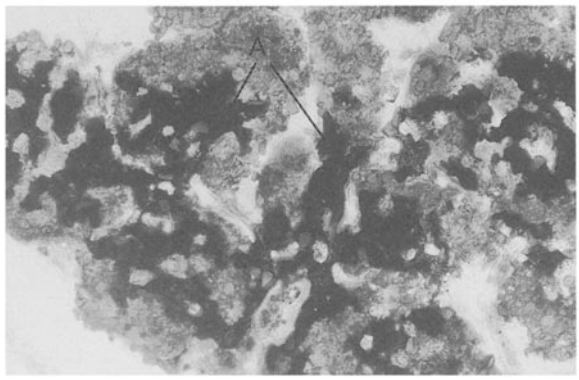

Figure 6 Bone tissue (A) on granules in the active reactor centre 4 months after implantation. Masson trichrome, × 50.

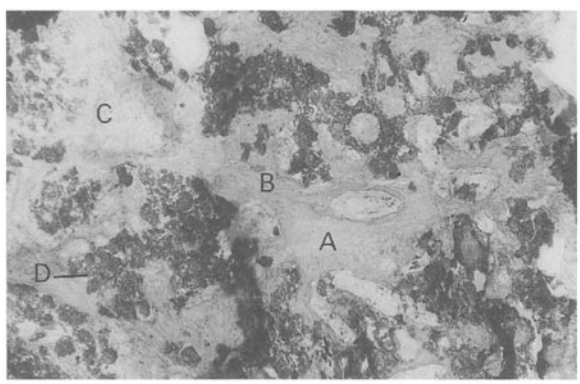

Figure 7 Mixture of bone tissue (A), osteoid substance (B), connective tissue (C) and HAP fragments (D) in the active reactor after 4 months. Masson trichrome, × 200.

reactor close to the interface (Fig. 4). The rest of the device was free of cells and filled with reticular fibres without any vascularization (Fig. 5).

After 2 months implantation, there was an ossification process in the active reactor and a conjunctive invasion in the control reactor as mentioned above.

At 4 months, ossification had progressed towards the centre of the active reactor (Fig. 6). The neoformed tissue was a mixture of fibrous tissue, cancellous bone, HAP granules and haemopoietic tissue (Fig. 7). At the bone–reactor interface there was bone tissue appending. Many HAP granules were fragmented, small fragments were phagocytozed by histocytes or fibroblast-like cells. On bigger fragments, osteoblasts synthesized osteoid and bone tissue. Vascularization could be seen in the entire reactor. Fluorescence examination of a reactor section showed a very high osteogenic activity.

The control reactor was surrounded by fibrous tissue, with a very poor loose connective tissue inside. Fluorescence examination did not show osteogenic activity.

The small number of samples and the great dispersion in the volume filled with new bone tissue in the active reactor did not allow an image analysis and statistical treatment of the sections as had been planned.

4. Discussion

Cells have been shown to fix very quickly by an adsorption process at the mineral surface. They synthesized a great amount of extracellular matrix immobilized on the surface. Cells grew in multilayers and bridged the granules with a very thick cellular and extracellular matrix rendering jointly liable granules [9].

The very high yield of the culture grown on this support allows a great cell quantity to be available. The osteochondrogenic potential of these cells was proved to persist after the culture [5]. The expansion of these lines by multiplication and immobilization on a biocompatible support which gave good handling properties allowed the use of this material as bone autograft material.

The use of a bioreactor permitted cell vascularization by medullar vessels. This vascularization is necessary for any ossification process. These previous and well-known results were confirmed by the fact that all bone tissue in the device was located in well-vascularized zones. Zones without any histological evidence of vascularization did not contain any bone tissue and sometimes no connective tissue. Cell nutrition by the diffusion process was active only in the first hours of implantation and did not lead in this case to any cell differentiation. This was different from the ossification in diffusion chambers which takes place without any vascularization of the tissue [3, 4].

The place of the granules in the volume of the reactor is not fixed. Therefore, the size of pores and the space between granules did not interact with tissue development like a macroporous block. Granules moved with the expansion of the new tissue.

This system did not allow granule fracture propagation from one granule to another.

Our previous findings showed that subcutaneous and intramuscular implantation of the same calcium phosphate combined with dog cancellous bone cells resulted in very irregular bone formation [10]. The proximity of bone tissue promoted a differentiation of stem cells towards osteogenic cell lines. However, at 4 months, all bone marrow cell lines could be found in the active reactor. In contrast, reactors which were not associated with stromal cells did not show ossification or tissue differentiation inside. The 2 cm cortical bone defect cannot heal without cell addition. HAP cannot furnish a support allowing tissue differentiation or tissue ingrowth from the edge of the implanted bone. The osteotrophic properties of HAP depended only on cell immobilization on its surface. Moreover, the biological influence of HAP inducing expression of some osteoblastic characteristics, such as alkaline phosphatase activity or proteoglycan synthesis *in vitro* [10], does not indicate osteoblastic phenotype induction, as shown by very inconsistent results obtained with immobilized stromal cells implanted in subcutaneous or muscular sites.

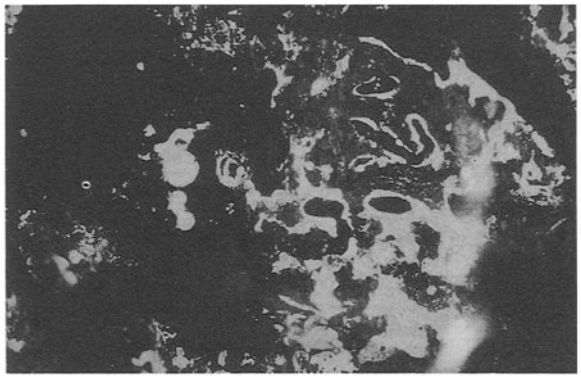

Figure 8 Tetracycline fixation in the centre of active reactor at 4 months, × 100.

Stromal cell immobilization on HAP provides an easy way to expand bone cell population and is a good means of obtaining applicable cell lineages for surgery. Immobilization may be as useful in an implantable microbioreactor as it has been in industrial reactors.

Bone obtained by this kind of cell graft is a woven bone quite similar to the bone obtained in long-bone fracture healing. We can reasonably predict that mechanical stress would lead to more compact bone. The yield of the reactor should be better by selection of a cell line with a greater percentage of more committed osteogenic cells. This implies that we could select using specific labelling molecules or antibodies, osteogenic-committed stem cells.

The ceramic on which the cells are immobilized has very poor mechanical properties. Structure, powder composition, size of particles and forming techniques of the ceramic carrier must be improved. The structure of such carriers must imperatively allow a good vascularization. The rate of ossification in an active reactor is not constant and the small number of reactors implanted did not permit a quantitative evaluation of ossification; however, the presence of bone tissue in active reactors and the absence of organized tissue in the control leads to the conclusion that autologous bone cell grafts to synthesize bone tissue in a large defect is possible [9].

Acknowledgements

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