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Incorporation of human osteoblast cells and osteoblast-like cells into porous hydroxyapatite scaffolds

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Abstract

The object of this study was to investigate methods of seeding cells onto porous hydroxyapatite granules with the aim of optimising cell attachment. Two cells types were used; an osteosarcoma cell line, MG63, and human osteoblasts (HOBs) isolated from trabecular bone. Several conditions were investigated to determine their effect on cell attachment. These included varying the initial seeding concentration, pre-adsorption of the granules with the adhesion protein fibronectin and the use of mechanical agitation. Human osteoblasts and MG63 osteosarcoma cells attached to both dense and porous HA granules but with a low seeding efficiency while seeding was not significantly improved by pre-coating scaffolds with fibronectin or by introducing fluid flow.

Introduction

Porous scaffolds are used for tissue engineering either on their own or as delivery vehicles for autologous cells isolated from the circulation and the bone marrow. They promote new tissue formation by providing a large surface area and pore volume to encourage the attachment, migration, proliferation and differentiation of cells throughout the region where new tissue is needed. Present research aims to use porous, bioactive, ceramic matrices as hosts for living cells. Following the growth of the cells *in vitro*, the 'living composite' can be transplanted into the patient. A bioactive matrix (e.g. porous hydroxyapatite) is required for the donor cells, isolated from bone marrow, for example, to develop an osteoblast-like phenotype and to produce a mineralised matrix with a mechanically strong interface with the porous scaffold.

Before this is attempted however, methods of seeding cells within the scaffolds need to be established in order to optimise cell attachment and to ensure an even distribution of cells within the scaffold. While others have used perfusion and vacuum assisted systems of cell seeding [1-3], this study aimed to investigate simple seeding methods while maximizing cell attachment. Two cell types were used; human osteoblast cells (HOBs) and a transformed osteosarcoma cell line, MG63. MG63s were used due to their high proliferative potential in order to produce large numbers of cells to maximise initial seeding densities.

Several conditions were used to determine their effect on cell attachment. These included varying the initial seeding concentration, coating granules with the adhesion protein fibronectin, and three different seeding techniques. These are outlined below.

Materials and Methods

Porous phase-pure hydroxyapatite (HA) granules (Apapore™, Apatech®, London, U.K.) with diameters between 2mm and 5mm were used in this experiment. Dense phase-pure HA granules of the same diameter were used for comparison. Several porous granules were also pre-soaked in a solution of fibronectin (100μg/ml in phosphate buffered saline) for 16 hours at 4° C, and air-dried in a sterile atmosphere for 2 days.

Experiment 1
Human osteoblast cells (HOBs), isolated from femoral heads, were grown in McCoy's medium supplemented with 10% v/v foetal calf serum, 2mM glutamine containing penicillin and streptomycin and Vitamin C (30g/ml). Cells at passage 6 were seeded at $5x10^4$ cells per porous HA granule by pipetting 250µl of cell suspension onto each granule in a well of a 96-well tissue culture plate and were incubated at 37°C, 5% CO_2 for 4 hours to allow for attachment. After this period, the granules were removed and each placed in a well of a 48-well plate, covered with 500µl of supplemented medium and incubated for a further 24 hours.
Experiment 2
MG63s were cultured in Dulbecco's modified Eagle medium supplemented with 10% v/v foetal calf serum and 2mM glutamine. Cells were seeded onto both porous and dense scaffolds with and without a fibronectin coating at 3 different initial seeding concentrations; $2.5x10^6$, $5x10^6$ and 10^7 cells per granule as described above, except that during attachment, the plate was placed on an orbital shaker at a speed of 20 revolutions per minute for 4 hours. Samples were incubated for a further 24 hours in supplemented medium.
Experiment 3
MG63s were seeded into both porous and dense scaffolds with and without a fibronectin coating at 3 different seeding concentrations, $2.5x10^6$, $5x10^6$ and 10^7 cells per granule, using 3 different seeding methods. 1) As described in *Experiment 1,* 2) As for *Experiment 1* but the 96-well plate was initially placed on a plate shaker and incubated at 37°C, 5% CO_2 for 30 minutes. After which, the plate was removed from the plate shaker and incubated for a further 3 ½ hours. 3) Granules were placed on sterile Teflon sheets and 100µl of cell suspension was placed on top of each granule to form a droplet around the granule. These were placed in Petri dishes and incubated at 37°C, 5% CO_2. After 4 hours, all granules were removed and placed in a 48-well plate and covered with 1ml of supplemented medium.
After incubation for 24 hours, all samples in experiments 1, 2 and 3 were removed and cell number was determined by the measurement of adenosine triphosphate (ATP) using the ViaLight™ HS kit (LumiTech, Nottingham, U.K.). Cell numbers were read from a standard curve generated using a series of known cell densities.

Results
Experiment 1
After incubation, 840 ± 390 HOBs had attached to the porous HA granules. This represents 1.7% of the original seeding concentration.
Experiment 2

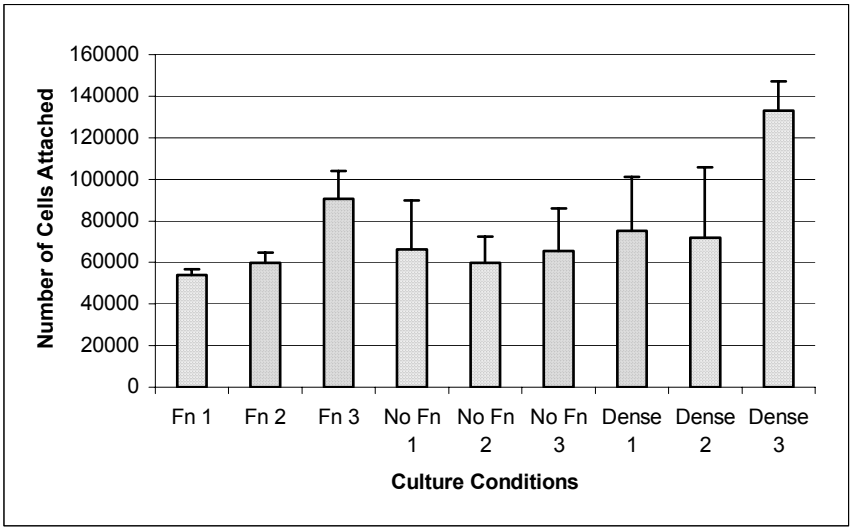

Fig. 1
Graph showing the number of MG63 cells attached to porous granules with and without fibronectin and on dense granules (mean, ± s.d., n=3)
Fn: Fibronectin
1: seeding density of $2.5x10^6$
2: seeding density of $5x10^6$
3: seeding density of $1x10^7$

Using higher seeding concentrations than for Experiment 1, between 0.9% and 3% of MG63s attached to the granules. The greatest number of cells attached to the highest seeding concentrations (Fig. 1) but these differences were not significant.

Experiment 3

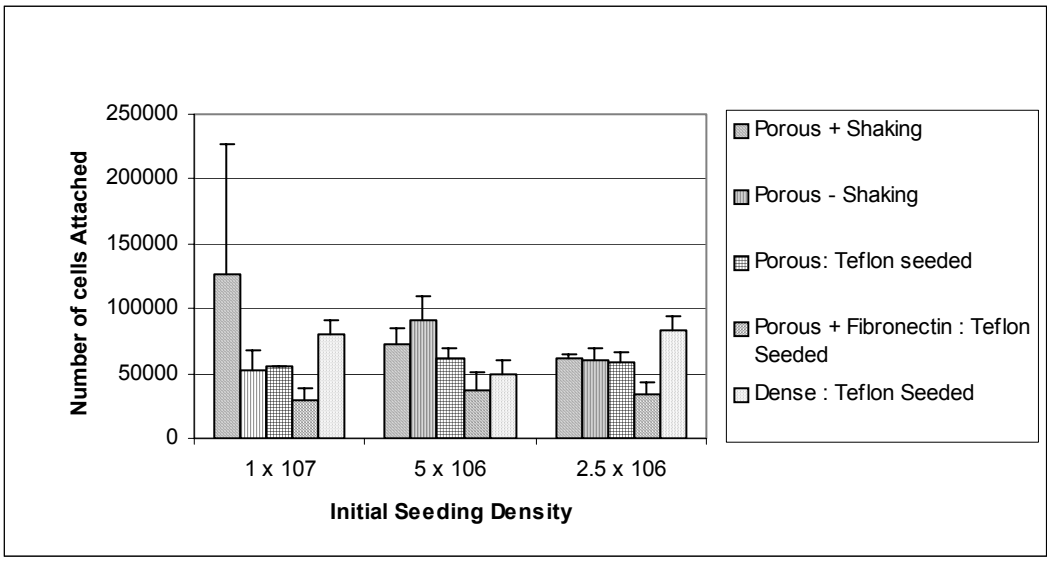

Fig. 2 Graph showing the number of cells attached to granules under various seeding conditions (mean, ± s.d., n=3)

Between 0.3% and 3.2% of MG63s attached to the granules. Significant differences were not seen between different initial seeding concentrations or by varying the seeding methods.

Discussion

The porous nature of the scaffold provides the implant with two critical functions. Firstly, the pore channels allow the entry of migrating cells and the development of capillaries, and secondly, they provide a large surface area for a number of specific cell interactions. A successful scaffold must allow tissue conduction. Therefore, cell attachment and migration within the scaffold must be promoted and proliferation and differentiation being encouraged [4].

There have been numerous studies of cell attachment and behaviour on various biomaterials [5-11]. Osteoblast adhesion has been found to be a consequence of the surface characteristics of the material; such as the topography, chemistry or the surface energy. The extent of attachment, adhesion and spreading of the cells on contact with the implant, influences the cell's capacity to proliferate and differentiate [6]. If the reality of tissue engineering is to be achieved, where a 'living composite' of biomaterial and autologous cells can replace damaged tissues, there must be a complete understanding of osteoblast adhesion to biomaterials in order to optimise the bone/biomaterial interface.

The aim of our study was to use a simple method of seeding cells into the porous HA granules while maximizing the cell number. We decided to load the granules with a high concentration of cells and combine this with gentle shaking during loading to allow the cells to infiltrate the inner pores of the granules. Previously we had found that when cells were seeded onto granules in tissue culture plastic plates, cells preferentially attached to the plate surface. In order to prevent this problem, the granules were seeded on hydrophobic Teflon sheets, so that the cells would attach preferentially to the porous HA substrate.

Using HOBs, 1.7% of cells attached to the porous granules and when higher seeding concentrations were used, between 0.3% and 3.2% of MG63s attached to the granules. The greatest number of cells attached at the highest seeding concentrations but there was no significant increase between $2x10^6$ and $1x10^7$ cells. It is possible that lower seeding concentrations are optimal. The use of shaking and fibronectin did not appear to increase cell attachment and the similar levels of attachment between the dense and porous granules did not reflect the increased surface area of the

latter. The results from Experiment 3, suggests that seeding the granules on a hydrophobic surface does not appear to increase cell attachment.
There have been several studies that have used flow perfusion or dynamic cell seeding systems to enhance cell incorporation into scaffolds. Van den Dolder et al [2] used a method of flow perfusion to seed marrow stromal osteoblasts in a titanium fibre mesh in comparison to a static method. They found that the meshes subjected to flow perfusion culture were completely covered with layers of cells and mineralised matrix while those cultured under static conditions only had a thin sheet of matrix on the upper surface of the mesh. Xiao et al [3] found a combination of dynamic and static methods was the most efficient in seeding fibroblasts into porous polymer scaffolds and resulted in an improved neodermis formation following skin graft and Uemura et al [1] showed that a low pressure method and a perfusion culture system improved the activities of cells in the central areas of porous materials. In another study, van Wachem et al [12] used a novel vacuum seeding method for the fast application of an evenly distributed cell layer on porous vascular grafts. Their study concluded that the technique rapidly introduced a confluent layer of seeded cells onto the porous grafts, which could easily be used in the operating theatre pre-implantation.
Due to the success of these methods, we propose to investigate the use of perfusion and vacuum seeding methods in future experiments.

Conclusions

- Human osteoblasts and MG63 osteosarcoma cells attached to both dense and porous HA granules but with a low seeding efficiency.
- Seeding was not significantly improved by pre-coating scaffolds with fibronectin or by mechanical agitation.
- Increases in seeding concentration produced only small increases in attachment.
- Further methods are required to optimise cell attachment to porous HA scaffolds. For example, using centrifugal force or perfusion.

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