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In vivo bone formation by human bone marrow cells: effect of osteogenic culture supplements and cell densities

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Bone marrow is known to contain a population of osteoprogenitor cells that can go through complete differentiation when cultured in a medium containing appropriate bioactive factors. In this study, porous particles of a calcium phosphate material were seeded with adult human bone marrow cells in the second passage. After an additional culture period of 1 wk in the particles, these hybrid constructs were subcutaneouslly implanted in nude mice with a survival period of 4 wk. The cell seeding densities range from 0–200 000 cells per particle and the cell culture system was designed to investigate the single and combined effects of dexamethasone and recombinant human bone morphogenetic protein 2 (rhBMP-2). The hybrid ''material/tissue'' constructs were processed for histology and the amount of *de novo* bone formation was quantified, for each culture condition, by histomorphometric techniques. The relative percentage of mineralized bone formation reached a maximal value of 19.77 $\pm$ 5.06, for samples cultured in the presence of rhBMP-2 and with a seeding density of 200 000 cells/particle, compared to 0.52 $\pm$ 0.45 for samples in which no cells had been cultured and had been incubated in culture medium supplemented with Dex and rhBMP-2. For the tested conditions and for the low cell numbers used in this study, rhBMP-2 proved to be an essential bioactive factor to obtain *in vivo* bone formation by our culture system. The results from this study prove the potential of cultured adult human bone marrow cells to initiate and accelerate *de novo* bone formation after transplantation into an ectopic site.

1. Introduction

In bone reconstructive surgery, the repair of critical-size bone defects is a major problem because the current therapies do not always provide an effective treatment. At present the use of autologous bone grafts is one of the most successful means of reconstruction. It avoids complications related to foreign body responses while providing bioactive molecules and cells that will allow effective regeneration. However, orthopaedic surgeons face substantial problems: bone is only available in limited quantities, the harvest procedure has associated health risks such as donor site morbidity and pain. These drawbacks motivated research activities and the tissue-culturing technology has emerged as a promising approach to treat these types of defects, without the limitations of the traditional therapies. In this approach, based on the combination of material science and biotechnology, cells are taken from a small biopsy, expanded *in vitro* and then seeded on to a biomaterial specially designed for this purpose. Then, cells are induced to follow osteogenic differentiation and finally transplanted into a patient bone defect to create new bone tissue.

The scaffolding biomaterial for tissue engineering purposes, obviously must be non-toxic, osteoconductive, allow for the attachment of cells and provide an adequate environment for their proliferation and for the ingrowth of vascular tissue, ensuring the survival of the transplanted cells.

A suitable site to harvest osteogenic cells is bone marrow; as marrow tissue has long been recognized as a source of osteoprogenitor cells that can be induced to differentiate along the osteoblastic lineage, when cultured under conditions permissive for the osteogenic development [1–4]. Furthermore, it has been claimed that cell populations from marrow tissue contain osteoprogenitors with more proliferative ability and greater capacity for differentiation than those originated from other skeletal sites [5].

Several investigators have demonstrated that cells grown from non-human marrow sources can be induced to osteogenically differentiate in response to

various bioactive factors including the synthetic glucocorticoid dexametasone [1, 2, 6–9], and rhBMP-2 [6, 10, 11]. Moreover, it was found that dexamethasone enhances the effect of rhBMP-2 on the differentiation of rat bone marrow cells and rat calvaria cells [6, 12, 13]. A drawback from these studies, however, is that the data are difficult to extrapolate to humans because the results from the *in vivo* experiments indicate that only non-human, and not adult human bone marrow cells, are able to form bone tissue [14–16].

The current investigation was designed to study the effects of the osteogenic supplements, dexamethasone and rhBMP-2, and cell seeding densities on the *in vivo* bone induction by adult human bone marrow cells. These cells were cultured for 1 wk in a porous ceramic biomaterial, to allow bone matrix formation, and afterwards, subcutaneously implanted into the back of nude mice.

2. Materials and methods

2.1. Materials

Porous granules of coraline hydroxyapatite (Pro-Osteon 500 particles) were obtained from Interpore. The interconnected pores had a median diameter of 435 μm and the size of the implanted particles was approximately 3 mm × 2 mm × 2 mm.

2.2. Human bone marrow cell (HBMC) isolation and culture

Cells were obtained from a 66 y old female patient undergoing total hip arthroplasty. After the removal of the femoral head, cancellous bone plugs of approximately 1 cm^3 were removed and transported in cold culture medium. Prior to further processing for the culture, the marrow cells were isolated by placing the plugs in 50 ml syringes, followed by repeated washing with culture medium until the bone plugs changed color from red to whitish. The cell suspensions were resuspended with a 20 G needle and then centrifuged, for 10 min, at 500 *g*. The resulting cell pellet was resuspended in α-MEM (Gibco, BRL) supplemented with 10% of foetal bovine serum (FBS) and antibiotics (culture medium) and finally plated in T75 flasks (one plug per flask). At near confluency, cells were enzymatically lifted from the flask using a 0.25% trypsin solution and cell counting was performed. The cells were then concentrated by centrifugation at 500 *g*, during 10 min, and the resulting pellet was resuspended in culture medium. Aliquots of 100 μl of cell suspension, containing 0, 50 000, 100 000 and 200 000 cells, were seeded in Pro-Osteon particles, placed in 24-well bacteriological grade plates. The cells were allowed to settle for 3 h, after which an additional 2 ml culture medium, supplemented with 50 $\mu g\,ml^{-1}$ ascorbic acid and 10 mM β-glycerophosphate, was added to each well. In order to evaluate the effect of osteogenic supplements, dexamethasone (Dex, 10^{-8} M) and/or rhBMP-2 (1 $\mu g\,ml^{-1}$) were also added to the medium. The cells were cultured for 7 d prior to implantation, to allow the production of an *in vitro* formed extracellular matrix. During that period, the culture medium was refreshed once. The cell seeding densities used for each condition were 0, 50 000, 100 000 and 200 000 cells per particle and triplicate samples were used per condition ($n = 3$).

2.3. *In vivo* implantation

Prior to implantation, the samples were soaked in serum-free medium and then in sterile phosphate buffered solution, pre-warmed to 37 °C. The nude mice were anaesthetized by an intramuscular injection of a mixture 2:6:7 atropine (67 $\mu g\,ml^{-1}$), xylazine (8 $mg\,ml^{-1}$) and ketamine (46.7 $\mu g\,ml^{-1}$). The surgical sites were cleaned with 70% ethanol and subcutaneous pockets were created in each side of the spine (two per side) in which the samples were implanted. At the end of the 4 wk survival period, the implants were removed and fixed in 1.5% glutaraldehyde in 0.14 M cacodylic acid buffer, pH 7.3.

2.4. Histological preparation

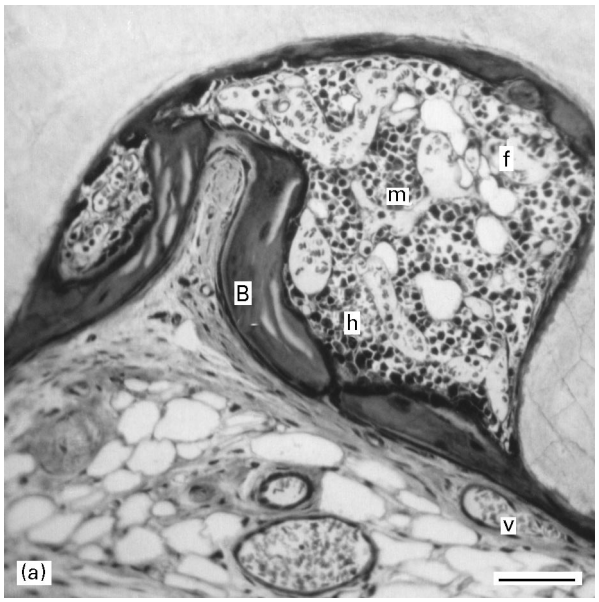
The fixed samples were dehydrated in increasing ethanol solutions to 100% ethanol and embedded in methyl methacrylate for sectioning. Approximately 10 μm thick, undecalcified sections were processed on a histological diamond saw (Leiden microtome cutting system). The sections were stained with basic fuchsin and methylene blue, in order to study bone formation. Micrographs of the stained samples were taken with a Leitz light microscope.

2.5. Histomorphometry

On all implants, the percentage of *de novo* bone formation was determined using a computerized image analysis system (VIDAS). The percentage of bone formation was calculated as the total surface area of bone in relation to the total surface area of implanted ceramic material. Although this measuring technique is not optimal, in the way that the obtained absolute values do not give information about the amount of formed bone as compared to the amount of pores within the implant, it provides a valid method to compare bone formation induced by the HBMC cultured in several different conditions. Furthermore, it follows measurement of not only the bone formed within the pores, but also bone formation on the outer surface of the implant.

3. Results and discussion

After 4 wk of implantation, all the constructs with cells grown in the presence of both rhBMP-2 and Dex produced osteogenic tissue. This comprised a bone matrix with embedded osteocyte cells and layers of osteoblasts. Cells cultured in the presence of rhBMP-2 but in the absence of Dex, only exhibited bone formation for the cell seeding densities of 100 000 and 200 000 cells. For both conditions, ingrowth of vascular tissue was observed adjacent to the bone, supplying the metabolic requirements to the newly formed bone. Moreover, bone marrow which included



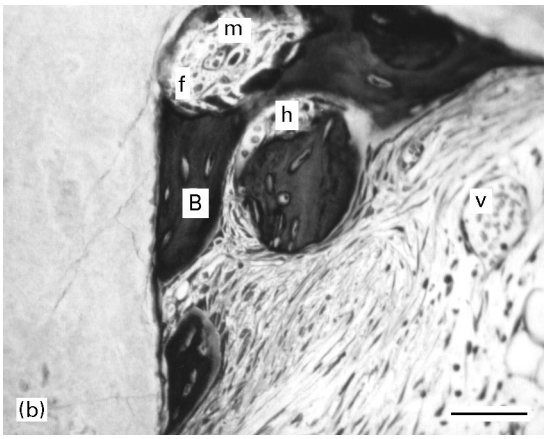


Figure 1 Bone formation by HBMC cultured in the presence of Dex and (a) rhBMP-2 and (b) rhBMP-2, after subcutaneous transplantation in nude mice for 4 wk. New bone shows osteocytes embedded within the matrix (B) and surrounds a bone marrow cavity (m) containing haematopoietic tissue (h) and fat cells (f). Blood vessels (v) are frequently observed near to bone.

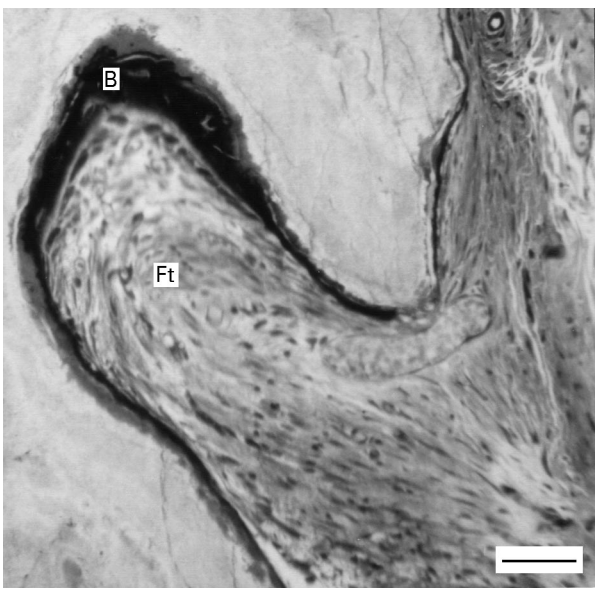


Figure 2 Bone formation after transplantation of the ceramic material soaked for 1 wk in rhBMP-2 and Dex containing medium. The thin bone line (B) formed at the implant surface is surrounded by fibrous tissue (Ft) with no bone marrow tissue.

blood vessels, fat and hematopoietic cells was also found in these implants (Fig. 1a and b).

Control samples, devoid of cultured cells, soaked in Dex plus rhBMP-2 containing medium infrequently revealed traces of bone tissue. A very thin and discontinuous layer of bone was sometimes detected near to the implant surface (Fig. 2). However, no marrow was ever found and the amount of bone formation was drastically less compared to implants with cultured cells, as proved by the histomorphometric measurements (Fig. 3). The percentage of bone in the implants devoid of cultured cells had an average value of 0.52 ± 0.45 and the addition of 50 000 cells to this same system lead to an average 23-fold increase in the percentage of bone production.

Several researchers [17–19] reported substantial ectopic bone formation by rhBMP-2 to which was associated the production of rich bone marrow. However, the concentrations of rhBMP-2 used in those studies were significantly higher than the concentration we used in our work. In this report, the lack of marrow tissue formation in control samples, soaked in medium with rhBMP-2 plus Dex, is probably related to the very small amount of newly formed bone. Therefore, this bone is not active enough to induce marrow production in the same time period. Interestingly, in the control samples, only rhBMP-2 (no Dex) gives no *de novo* bone formation, indicating that the combination of these two bioactive factors seems to result in a synergetic mechanism which leads to bone formation.

All the constructs cultured in control media (without rhBMP-2 and Dex) produced only fibrous tissue with no signs of bone (data not shown), revealing that the complete differentiation of osteoprogenitor cells contained in the adult human bone marrow needs to be potentiated by bioactive factors.

These findings seem to be in good agreement with those of several other authors [2, 17, 18, 20, 21], who reported the inductive potential of rhBMP-2 in the differentiation of osteoprogenitor cells from animal and human origin. Furthermore, for both control samples (without cells) and samples with the lowest seeding density, Dex appears to potentiate the osteoinductive effect of rhBMP-2. However, for higher cell seeding densities, the presence of Dex leads to a significant decrease in the extent of bone formation (Fig. 3). These results are indicative that, after 1 wk culturing, the amount of osteoprogenitor cells was lower in samples cultured in the presence of Dex, which may be due to a proliferation-delaying effect caused by Dex over the cells. This also would explain the lack of bone formation on implants cultured in the presence of Dex and the absence of rhBMP-2. The synergetic mechanism detected between the two bioactive factors, for the lowest cell density, can be due to a balance between the proliferative effect of rhBMP-2 and the non-proliferative effect of Dex. For higher amounts of osteoprogenitor cells, a higher concentration of rhBMP-2 would be needed to reach such a balance.

Some investigators [22] have already reported the *in vivo* bone formation by HBMC when cultured in

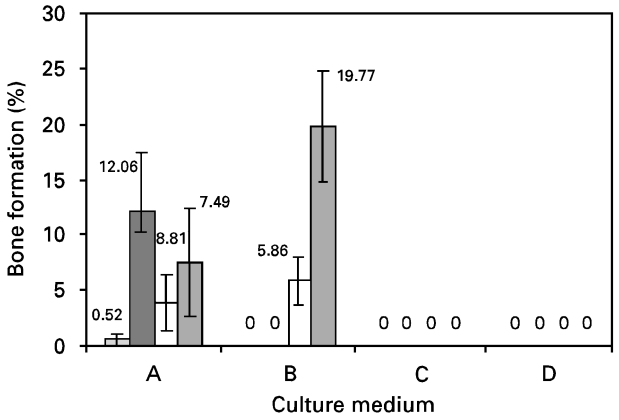


Figure 3 Bone formation by adult HBMC: effect of osteogenic supplements and cell seeding densities. (A) + rhBMP-2 + Dex; (B) + rhBMP-2 − Dex; (C) − rhBMP-2 + Dex; (D) − rhBMP-2 − Dex. □ Cells/part. 0. ■ Cells/part. 5.00×10^4. □ Cells/part. 1.00×10^5. ■ Cells/part. 2.00×10^5.

the presence of Dex. However, the cell densities used in these studied were substantially higher and those cells were from young-patient origin, having therefore a much higher proliferative potential than the adult HBMC that we described in this report.

Concerning the effect of cell seeding densities on *in vivo* bone induction by HBMC, we found that for implants cultured in rhBMP-2 containing medium, the amount of seeded osteoprogenitor cells has a direct and significant relation to the extent of formed bone. An increase of 100 000 seeded cells to 200 000 leads to a rise in the percentage of bone formation from 5.86 ± 2.14 to 19.77 ± 5.06. For samples cultured in a medium with a combination of rhBMP-2 and Dex, this relation is not so clear, and we observe a substantial decrease in the amount of formed bone when the cell seeding density increases from 0.5×10^5 to 1×10^5. These observations may be also related to the hypothesis that a higher amount of cells needs a higher concentration of rhBMP-2 to balance the proliferative delaying effect of Dex.

4. Conclusion

The ability to grow bone tissue that supports haematopoiesis, by adult HBMC was established in this study. These results are encouraging and indicate the regenerative potential of tissue culturing technology for bone reconstruction.

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