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Bone tissue engineering on calcium phosphate-coated titanium plates utilizing cultured rat bone marrow cells: a preliminary study

R. J. DEKKER*, J. D. DE BRUIJN‡, I. VAN DEN BRINK, Y. P. BOVELL, P. LAYROLLE, C. A. VAN BLITTERSWIJK
*University of Twente, and ‡Biomaterials Research Group, University of Leiden, The Netherlands and also at IsoTis BV, Professor Bronkhorstlaan 10, 3723MB Bilthoven, The Netherlands
E-mail: robert.dekker@isotis.com

The use of osteoinductive *in vitro* tissue-coated implants in orthopaedic and dental surgery (e.g. revision hip arthroplasty), could result in a better fixation of these implants. However, this tissue engineering technology has only proved to be effective in porous materials and not on flat implant surfaces. In this study we have demonstrated that calcium phosphate-coated titanium plates with a layer of cultured osteogenic cells and their extracellular matrix can initiate bone formation *in vivo*. Both primary and subcultured rat bone marrow cells were grown on to biomimetic calcium phosphate-coated titanium plates. After 7 d of culture, in the presence or absence of dexamethasone, the implants were subcutaneously implanted in nude mice for 4 wk. Control samples, which consisted of calcium phosphate-coated plates without cultured cells and porous calcium phosphate particles with or without cultured cells, were also implanted subcutaneously. At autopsy, no bone formation could be detected on any of the control samples without cells and samples with subcultured cells, which were primary cultured in medium without dexamethasone. In contrast, clear *de novo* bone formation could be observed on the calcium phosphate-coated plates and in the porous calcium phosphate particles with primary or subcultured cells, which had been continuously cultured in medium with dexamethasone. These results indicate that this hybrid technology offers great potential for the fixation of flat bone replacement implants (e.g. artificial hips) in inferior bone in the future.

1. Introduction

Hydroxyapatite ($Ca_{10}[PO_4]_6OH)_2$-coated titanium (Ti6Al4V) implants are widely used in orthopaedic and dental surgery [1, 2]. An example is the use of hydroxyapatite-coated artificial hips in primary hip arthroplasty, in which excellent results have been obtained. Geesink and Hoefnagels [1] even reported a survival rate of 100% of hydroxyapatite-coated stems, after 6 y implantation. In general, however, current cemented and non-cemented hip prostheses do not perform well in *revision surgery*, where they have a significant failure rate of 50% after 6 y implantation [3]. This high failure rate is, to a large extent, due to the presence of large bone defects around the revision hip implants, which cannot be adequately healed as a result of the current non-optimal bone filler materials. In this case, an implant which is not only bioactive but also osteoinductive, could offer great potential. The most ideal approach hereby, would be to coat the implant surface with a layer of autologous living bone tissue. Such a layer of cultured bone could be obtained by growing bone marrow cells under specific culture conditions to facilitate proliferation and differentiation of osteoprogenitor cells to bone-producing osteogenic cells. These cultured cells could subsequently be seeded on an implant surface and after an additional culture period, the tissue-coated implant could be implanted in patients. This hybrid, tissue engineering technology, which combines material technology and biotechnology, should eventually lead to an enhanced bone–implant integration.

The use of tissue-coated [4, 5] and cell-coated [6–8] implants has already proved to be successful in porous implants (e.g. porous hydroxyapatite). However, to our knowledge, nobody has shown that such bone tissue engineering on flat surfaces is possible. The development of this technology could be of great benefit for a better implant fixation in orthopaedic and dental surgery (e.g. revision hip arthroplasty). Therefore, the aim of this study was to examine whether cultured rat bone marrow cells and their extracellular matrix can initiate bone formation *in vivo*, when grown on to biomimetic calcium phosphate-coated

titanium plates. Cultured rat bone marrow cells in porous Interpore (calcium phosphate) particles served as controls, because these hybrid constructs have already proven to be osteoinductive [4].

2. Materials and methods

2.1. Materials

A biomimetic amorphous calcium phosphate (Ca/P) coating (approximately 0.5–15 μm thick) was produced by incubation of titanium (Ti6Al4V) plates (10 mm × 10 mm × 1 mm) in a simulated body fluid at 37 °C, according to Layrolle *et al.* [9]. The coating was not homogeneous in thickness, due to the preliminary stage of the coating procedure. Prior to use, all samples were sterilized by autoclaving for 20 min at 121 °C. Round porous Interpore (calcium phosphate) particles (Pro-Osteon 500) with an average pore diameter of 435 μm and a particle diameter of approximately 1.5 mm were used as controls. Prior to cell culturing, plates and particles were placed in 25-well bacteriologic culture plates (1 per well).

2.2. Cell isolation

According to a slight modification of the method described by Maniatopoulos *et al.* [10], bone marrow cells were obtained from six femora of 100–120 g young adult male Wistar rats. In order to synchronize the seeding times of primary and subcultured cells (see below), the femora of, respectively, 1 and 2 rats were isolated at two different time periods.

2.3. Cell culture and seeding of subcultured cells

The cells obtained from four femora were primary cultured in fully supplemented medium, that consisted of α-Minimal Essential Medium (α-MEM-RNA/DNA, Gibco) with 15% foetal bovine serum (FBS, Gibco), 0.1 mg ml^{-1} penicillin G (Boehringer-Mannheim), 50 mg ml^{-1} gentamycin (Gibco), 0.3 mg ml^{-1} fungizone (Gibco) and freshly-added 10 mM β-glycerophosphate (Gibco), 0.1 mM L-ascorbic acid-2-phosphate (Sigma), with or without 10 nM dexamethasone (Dex; Sigma) (see Table I). The medium was changed after the first 24 h to remove non-adherent cells and was further refreshed after 4 d culturing. After 7 d (at near confluency) the cells were detached with 0.25% trypsin containing 1 mM EDTA (Sigma) and pooled to obtain uniform cell population. The harvested cells were subsequently centrifuged and resuspended in α-MEM with 15% FBS and antibiotics to obtain a cell suspension of 5×10^5 cells/150 μl. This cell suspension was seeded as droplets (150 μl) on to the Ca/P-coated titanium samples and the Interpore particles, in order to inoculate 5×10^5 cells per sample. After 3 h, 2 ml fully supplemented medium, with or without Dex (see Table I), was added to each well containing either a Ca/P coated titanium sample or an Interpore particle. Respectively, 2 and 4 d after seeding the cells on the materials, the medium was refreshed.

TABLE I Overview of the number of samples used in this study. The signs − Dex/ + Dex, etc., represent the absence or presence of 10 nM Dex in the culture medium of, respectively, primary and subcultured cells

	Ca/P Ti plates (*in vivo*)	Interpore (*in vivo*)
Primary cells 1/10th (+ Dex)	4	4
Primary cells 1/20th (+ Dex)	4	4
Subcultured cells (+ Dex/ + Dex)	4	5
Subcultured cells (− Dex/ + Dex)	4	2
Subcultured cells (− Dex/ − Dex)	4	2
Without cultured cells (+ Dex/ + Dex)	4	4

2.4. Cell culture and seeding of primary cells

The cells obtained from the femora, which were used to seed RBMCs directly on to the Ca/P-coated titanium samples and the Interpore particles, were centrifuged and resuspended in 3 ml α-MEM with 15% FBS and antibiotics. Subsequently, droplets of 150 μl were seeded directly on to the Ca/P-coated titanium samples and the Interpore particles (see Table I), in order to seed 10% of the bone marrow volume that is present in one femur. The cell suspension which was left, was diluted with an equal volume of culture medium and 150 μl (5% of the bone marrow volume of one femur) was seeded on to the samples according to Table I. After 3 h, 2 ml fully supplemented medium (see above) with Dex was added to the wells. The medium was changed after the first 24 h to remove non-adherent cells and was further refreshed after 4 d culturing. Control samples, which consisted of Ca/P-coated plates and Interpore particles without cultured cells, were also placed in fully supplemented culture medium with Dex. All cultures (primary and subcultures) were incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C.

2.5. Implantation procedure

The Ca/P-coated plates and Interpore particles were implanted in the backs of 12 wk old nude mice (Balb/cOla Hsd-nu mice; approximately 25 g) after 7 d *in vitro* culturing. The mice were anaesthetized by an intramuscular injection of 0.04 ml Ketamine (46.7 μg ml^{-1}), Xylazine (8 mg ml^{-1}) and Atropine (67 μg ml^{-1}) mix. The skin was cleaned with 10% ethanol/iodine and two subcutaneous pockets were created on each lateral site of the spine of the back of the mouse. One Ca/P-coated plate or two to three Interpore particles were inserted in each pocket. In total nine mice were used for implantation.

2.6. Light microscopy

Four weeks post-operatively, the mice were sacrificed by cervical dislocation. The *in vivo* samples with

surrounding subcutaneous tissues, were removed and fixed in 1.5% glutaraldehyde in 0.14 M cacodylate buffer for at least 24 h at 4 °C. The samples were dehydrated through a graded series of ethanol and embedded in methyl methacrylate (MMA). Histological sections with a thickness of approximately 10 μm were cut on a modified innerlock diamond saw, stained with methylene blue and basic fuchsin and observed under light microscopy.

2.7. Backscatter electron imaging

Backscatter electron imaging was performed on unstained light microscopical blocks, which had been polished with 4000 grit silicon carbide sandpaper. The blocks were subsequently cleaned with 70% ethanol, placed on specimen holders and coated with a layer of carbon (Balzers). Samples were examined in a Philips S525 scanning electron microscope at 20 kV in backscatter mode.

3. Results

3.1. Ca/P-coated titanium plates

After 4 wk implantation, no bone formation could be detected on any of the control samples without cultured cells. The implanted plates with subcultured cells, which were primary cultured in medium without Dex and subcultured in medium with or without Dex, did not show any bone formation. In contrast, bone formation was detected at a significant surface area of the hybrid structures, which contained primary (10% and 5% of bone marrow volume of one femur) or subcultured cells and were continuously cultured in medium with Dex (Table II and Figs 1 and 2). Although there was morphologically little difference between the bone formation of these different constructs, two of the four constructs with subcultured cells showed bone formation, whereas all samples with primary cells revealed *de novo* bone formation. The newly formed layer was either unorganized, immature woven bone with many osteocytes (Fig. 1) or was more organized, which resulted in a dense layer of bone (Fig. 2). Both the unorganized and the organized layer of bone could be detected on Ca/P-coated plates with primary and subcultured bone marrow cells (continuously cultured in the presence of Dex). The layer of bone was up to 100 μm thick and showed osteocytes and a seam of secretory osteoblasts. The layer of newly formed bone was usually in direct contact with the plate and could only be detected at the Ca/P-coated site of the implants. Although backscatter electron imaging (Fig. 3) revealed that the coating was partially absent, the newly formed bone was usually associated with an intact layer of the Ca/P coating. However, *de novo* bone formation on areas without Ca/P coating could also be observed.

3.2. Control Interpore particles

Histological sections revealed fibrous tissue invasion with vascularization into the porous regions of each implant, after 4 wk implantation. Similar to the

TABLE II Overivew of the results obtained in this study. The signs − Dex/ + Dex, etc., represent the absence or presence of 10 nM Dex in the culture medium of, respectively, primary and subcultured cells. This table represents the number of samples with *de novo* bone formation *in vivo* versus the total number of samples per culture condition

	Ca/P Ti plates (*in vivo*)	Interpore (*in vivo*)
Primary cells $1/10^{th}$ (+ Dex)	4/4	4/4
Primary cells $1/20^{th}$ (+ Dex)	4/4	4/4
Subcultured cells (+ Dex/ + Dex)	2/4	5/5
Subcultured cells (− Dex/ + Dex)	0/4	0/2
Subcultured cells (− Dex/ − Dex)	0/4	0/2
Without cultured cells (+ Dex)	0/4	0/4

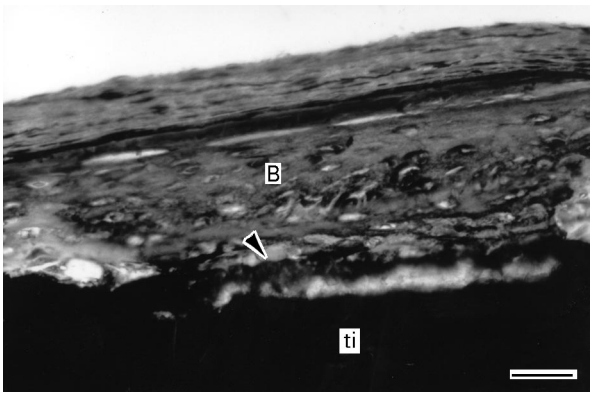

Figure 1 Light micrograph of bone (B) formation on a Ca/P-coated titanium plate, after 4 wk implantation. Prior to implantation, primary RBMCs (10% bone marrow volume of one femur) had been cultured on the plate for 1 wk in Dex-containing medium. Note the many osteocytes and the Ca/P coating (arrow) on the titanium (ti) surface.

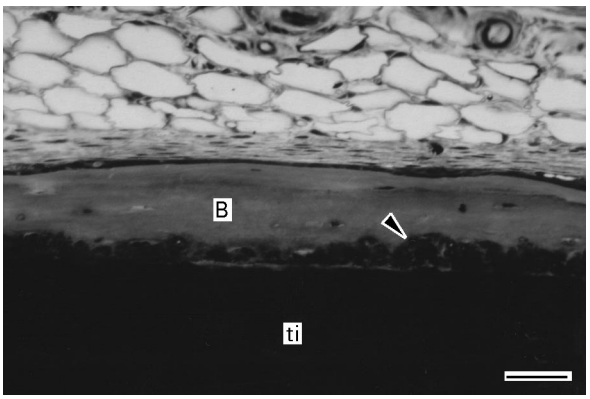

Figure 2 Light micrograph of bone formation on a Ca/P-coated titanium plate (ti), after 4 wk implantation. Prior to implantation, subcultured RBMCs (primary cultured in medium with Dex) had been cultured on the plate for 1 wk in Dex-containing medium. Note the newly formed bone (B) on the Ca/P coating (arrow), which is denser compared to the layer of bone-like tissue presented in Fig. 1.

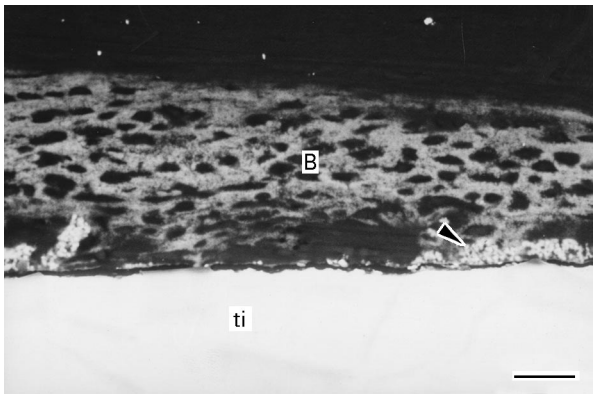

Figure 3 Back-scattered electron image of mineralized bone (B) on a Ca/P-coated titanium plate (ti), after 4 wk implantation. Prior to implantation, primary RBMCs (10% bone marrow volume of one femur) had been cultured on the plate for 1 wk in Dex-containing medium. Note the direct contact of the *de novo* bone with the Ca/P coating (arrow) at spots where the coating was present. Also note the areas were the coating was absent.

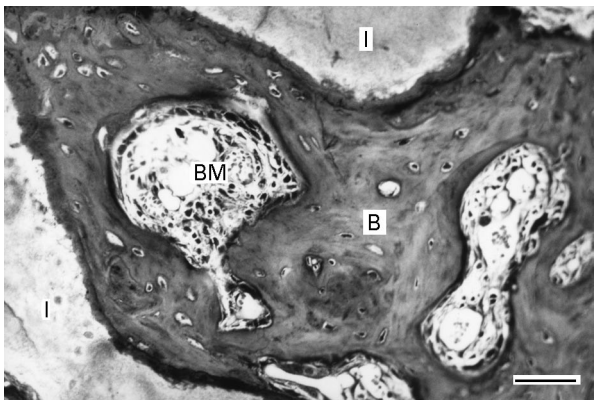

Figure 4 Light micrograph of bone formation in an Interpore particle, after 4 wk implantation. Prior to implantation, subcultured RBMCs (primary cultured in medium with Dex) had been cultured in the particle for 1 wk in Dex-containing medium. Note the direct bone contact of the newly formed bone (B) with the Interpore (I) surface and the bone marrow (BM) formation in the center of the newly formed bone.

Ca/P-coated plates, none of the samples without cultured cells or with cultured cells, which were primary cultured without DeX, showed any bone formation. However, after 4 wk implantation, clear bone formation at the periphery and in the pores of the hybrid structures could be detected in all the samples which contained primary (10% and 5% of the bone marrow volume of one femur) or secondary cells and were cultured only in medium with Dex (Table II and Figs 4 and 5). The newly formed bone was even present in the most central pores and was in direct contact with the Interpore surface. Bone marrow could often be observed in the center of this newly produced bone (Figs 4 and 5). The formed bone was organized and exhibited many osteocytes and a peripheral secretory seam of osteoblasts.

4. Discussion

Many authors have reported that cultured bone marrow cells (with or without extracellular matrix) in

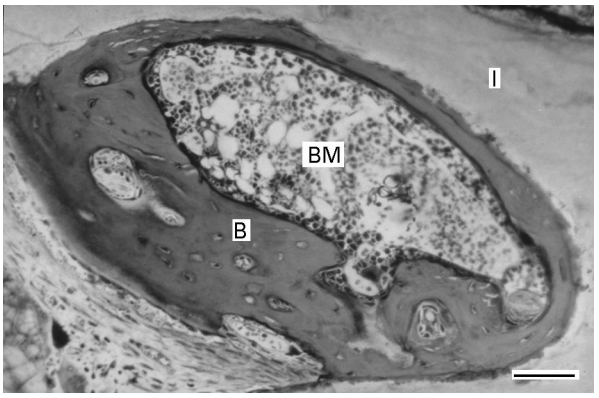

Figure 5 Light micrograph of bone formation in an Interpore particle, after 4 wk implantation. Prior to implantation, primary cultured RBMCs (10% bone marrow volume of one femur) had been cultured in the Interpore (I) particle for 1 wk in Dex-containing medium. Note the clear bone marrow formation (BM) in the center of the newly formed bone (B).

porous biomaterials, can induce bone formation after subcutaneous implantation [4–8]. However, the results presented herein show that cultured RBMCs and their extracellular matrix can initiate bone formation *in vivo*, when grown on flat biomimetic Ca/P-coated titanium plates. *De novo* bone formation on the Ca/P-coated plates could only be detected in constructs, which contained primary (10% and 5% of the bone marrow volume of one femur) or subcultured cells (5×10^5 cells). In addition, these cultured cells had to be continuously cultured in Dex-containing medium. Cells which were first primary cultured in medium without Dex, did not show any bone formation. This suggests that, in the absence of stimulatory agents for differentiation into the osteogenic lineage, the mesenchymal and/or osteoprogenitor cells of the primary culture had, respectively, differentiated directly or indirectly (after dedifferentiation) into another mesenchymal tissue (e.g. fibroblasts). When Dex was eventually added to the subculture medium, a lack of undifferentiated cells could have resulted in the shortage of the minimal number of osteoblasts, which are required for *in vivo* osteoinduction after subcutaneous implantation.

The Ca/P coating on the titanium plates was partially absent after 4 wk implantation. This was probably due to the experimental stage of the coating procedure, which resulted in a non-homogeneous coating. Other reasons for the partial absence of the coating could be degradation or detachment of the coating. This will be examined in more extensive studies in the future. Nevertheless, the newly formed bone was usually associated with an intact coating, although *de novo* bone formation could also be observed in areas devoid of a Ca/P coating. Hott *et al.* [11] reported that osteoblastic cells attach, proliferate and differentiate on hydroxyapatite with a sequence similar to that of culture-plastic in short-term cultures. These features, together with the fact that hydroxyapatite has a bone-bonding ability, makes Ca/P-coated materials very suitable for tissue engineering on flat surfaces of load-bearing implants. In contrast, porous materials used for bone tissue engineering of

non-loaded louded filling implants, do not necessarily need this bone-bonding ability. These materials, however, do need the ability to obtain cell attachment, proliferation and differentiation.

The pattern of bone formation in the *in vivo* control samples which consisted of cultured cells and porous Interpore particles, was the same compared to the Ca/P-coated plates in combination with cultured cells. Again, only the RBMCs cultured in the continuous presence of Dex could induce bone formation after subcutaneous implantation. These results indicate that continuous Dex-treated RBMCs are osteoinductive, when grown on a suitable biomaterial and implanted subcutaneously in nude mice. Also other culture conditions have proved to be successful in differentiating progenitor cells into osteoblasts. Several researchers have demonstrated that recombinant human bone morphogenetic protein-2 (rhBMP-2) is involved in the differentiation of osteoblast precursor cells into mature osteoblasts [12, 13]. It has furthermore been shown, that rhBMP-2 can contribute to *de novo* bone formation at ectopic sites, when added to the culture medium of porous calcium phosphate/bone marrow cell hydrides before implantation [14, 15].

Ca/P-coated plates in combination with primary cultured RBMCs, showed more frequently *de novo* bone formation than in combination with subcultured cells (see Table II). This difference could not be seen in the Interpore particles, which contained primary or subcultured cells (continuously cultured in medium with Dex). These more constant results obtained with the porous Ca/P particles, could be due to the presence of a micro-environment, which was created by the three-dimensional architecture of the particles. This micro-environment could enhance *de novo* bone formation, although these results have demonstrated that a three-dimensional architecture is not required for osteoinduction. Another explanation could be that a possible non-homogeneous Ca/P coating on the titanium plates could have resulted in less cell attachment, proliferation and differentiation. Also the initial seeding concentration of osteoprogenitor cells per square centimeter on the porous particles could have been higher compared to the cell seeding concentrations per square centimeter on to the plates, due to a possible bigger total surface area of the plates.

Despite the better results obtained with the primary cells compared to the subcultured cells in combination with the plates, the use of subcultured cells would be preferable when this technology would be used clinically. The ability to obtain enough bone marrow with the requisite number of osteoprogenitor cells could be a problem when primary bone marrow cells are used for seeding on implants. This indicates that the optimization of the culture technique for expanding and differentiating bone marrow cells would be of great benefit. The final goal is to optimize the bone culture process for humans and to use this technique for the fixation of flat bone replacement implants in inferior bone.

5. Conclusion

This is, to our knowledge, the first time that this hybrid, tissue engineering technology has been shown to be effective on flat surfaces. This offers great potential for the fixation of flat bone replacement implants in inferior bone in the future (e.g. for revision hip arthroplasty).

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