Isolation and osteogenic differentiation of rat periosteum-derived cells

Heidi Andrea Declercq, Leo Isabelle De Ridder and Maria Jozefa Cornelissen*

*Department of Anatomy, Embryology, Histology and Medical Physics, Louis Pasteurlaan 2, Ghent University, B-9000 Ghent, Belgium; *Author for correspondence (e-mail: Ria.Cornelissen@Ugent.be; phone: +32-9-2649240; fax: +32-9-2649498)*

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Abstract

Selection of appropriate cultures having an osteogenic potential is a necessity if cell/biomaterial interactions are studied in long-term cultures. Osteoblastic cells derived from rat long bones or calvaria have the disadvantage of being in an advanced differentiation stage which results in terminal differentiation within 21 days. In this regard, less differentiated periosteum-derived osteoprogenitors could be more suitable.

Periosteum-derived cells were isolated from the tibiae of adult Wistar rats ($n = 12$). The osteogenic potential with regard to alkaline phosphatase activity, morphology, nodule formation and mineralization was studied by culturing them in an osteogenic medium for up to 4 months.

Seventy-five percent of the cultures ($n = 9$) did not show any increase in alkaline phosphatase activity nor nodule formation during long-term culture for up to 4 months. Nevertheless, in 25% of the cultures, alkaline phosphatase activity started from negligible (< 5 mM pNP/mg protein) and increased towards approximately 50 mM pNP/mg protein. Three-dimensional nodule formation was observed at passages 3–5. In further passages (P5–P7), nodule formation capacity decreased and a diffuse mineralization pattern was observed. Suitable cultures with osteogenic capacity, can be selected at early passages based on the presence of cuboidal cells. These cells have the advantage of retaining their osteogenic potential even after prolonged cultivation (6–7 passages) before final differentiation occurs. Although periosteal cells are suitable for long term *in vitro* evaluation of biomaterials, the isolation and selection is time consuming. Hence, a more appropriate source to study cell/biomaterial interactions should be more convenient.

Introduction

In bone fracture healing, bone defects are restored by proliferation, migration and differentiation of cells derived from the inner layers of the periosteum. During embryonic and postnatal bone growth, these osteoprogenitor cells directly differentiate into bone forming osteoblasts. During fracture healing however, osteogenesis as well as chondrogenesis is observed. In this process of

healing, direct differentiation of the progenitor cells into bone forming cells is preferable. It is known that local micro-environmental conditions may be important in directing the differentiation of the periosteum-derived cells towards either osteogenesis or chondrogenesis. As such, periosteal cells could be used as an *in vitro* culture model to study the osteogenic capacity of biomaterials developed for bone regeneration therapy.

In order to study cell/biomaterial interactions, cells obtained from calvaria (Attawia et al. 1995; Burdick et al. 2002; Dalby et al. 2002; Moursi et al. 2002) and long bones (Scotchford et al. 1998; Calandrelli et al. 2002) are frequently used. Disadvantages of this type of cells are related to their advanced differentiation stage. Consequently, it is difficult to expand them into large amounts or to keep them in culture for long periods (Declercq et al. 2004; Salgado et al. 2004). The latter renders these cells less suitable for long-term *in vitro* test systems. The use of progenitor cells (periosteum or bone marrow derived cells) may circumvent these problems (Ishaug et al. 1997; Perka et al. 2000; Arnold et al. 2002; Spitzer et al. 2002; Lisignoli et al. 2001).

Periosteum-derived cells are in a less differentiated stage, which may favor easier expansion and maintenance for longer periods. In the literature, several attempts to culture and differentiate periosteum-derived cells towards the osteogenic lineage were made (Koshihara et al. 1987, 1989; Breitbart et al. 1998; Solchaga et al. 1998). Some studies to evaluate the osteogenic potential of biomaterials were performed which used periosteum-derived cells from human, rabbit, pig and bovine origin (Perka et al. 2000; Arnold et al. 2002; Spitzer et al. 2002). However, rat periosteum-derived cells were not yet analyzed with regard their suitability to study cell/biomaterial interactions.

The aim of the present study is to analyze if rat periosteum-derived cells can be easily harvested and expanded *in vitro* and if these cells have a high probability to differentiate along the osteogenic lineage. In this study, periosteum from adult rat long bones was harvested, expanded and analysis of the differentiation potential into the osteogenic lineage was performed. Osteogenic differentiation was evaluated based on alkaline phosphatase activity (an early marker), morphology, nodule formation and mineralization of a collagen containing extracellular matrix (late markers).

Materials and methods

Materials

Cell culture

DMEM glutamax-1 (low glucose) (Cat no. 21885-025), fetal bovine serum (FBS heat inactivated, E.C. approved), α -MEM supplemented with nucle-

otides (α -MEM DNA/RNA) (Cat no. 22571-020), L-glutamine, penicillin–streptomycin (10,000 U/ml–10,000 μ g/ml) and Fungizone[®] were purchased from Gibco BRL (Life Technologies, Merelbeke, Belgium). L-Ascorbic acid 2-phosphate and β -glycerophosphate were supplemented from Sigma (Sigma–Aldrich NV/SA, Bornem, Belgium). Tissue culture dishes and Thermanox[®] coverslips were purchased from Nunc (Life Technologies, Merelbeke, Belgium).

Assays

p-Nitrophenylphosphate, *p*-nitrophenol, glycine, silver nitrate and Tween 20[®] were purchased from ICN Biomedicals, Inc. (Asse-Relegem, Belgium). Fast Red TR/Naphtol AS-MX, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system, peroxidase substrate and collagen (C-8886) were from Sigma (Sigma–Aldrich NV/SA, Bornem, Belgium). The protein assay kit and bovine gamma globulin standard was supplemented from Bio-Rad Laboratories (Nazareth-Eke, Belgium). Bovine serum albumin (fraction V) was purchased from Boehringer Mannheim (Roche Diagnostics, Brussels, Belgium). Normal swine serum, biotinylated swine anti rabbit serum, peroxidase-conjugated streptavidin and Dako[®] Faramount aqueous mounting medium were supplemented from Dako Diagnostics (NV DakoCyomation, Heverlee, Belgium). Rabbit anti rat collagen I was from Institut Pasteur de Lyon. Sodium thiosulphate was purchased from Merck (VWR International, Leuven, Belgium). $K_4Fe(CN)_6 \cdot 3H_2O$ and Canada balsam were obtained from Vel (VWR International, Leuven, Belgium).

Isolation and culture of periosteum-derived cells

Periosteal explants were harvested from the tibiae (hind limbs) of 12 adult (3 month old) Wistar rats. The skin and underlying muscular-fibrous connective tissue were removed to expose the periosteum. The periosteum was stripped off mechanically using forceps, washed in a Ringer solution containing 0.5 vol.% penicillin–streptomycin and cut into flaps of 2 $\times$ 2 mm. The explants were placed with their osteogenic (cambium) side downwards into T25 tissue culture dishes (Bahrami et al. 2000; Hanada et al. 2001; Koshihara

et al. 1987, 1989) and were allowed to adhere to the tissue culture plastic (10 min, 37 °C) with the bottle placed upright before standard culture medium was added (Schwartz 1997). This medium consists of DMEM glutamax-1 (low glucose) + 10 vol.% fetal bovine serum + 0.5 vol.% penicillin–streptomycin + 1 vol.% Fungizone® + 100 µM L-ascorbic acid 2-phosphate.

After the cells near the explants had reached confluence, the monolayer was trypsinized, neutralized with fetal bovine serum and centrifuged (1000 rpm, 10 min). From then on, an osteogenic medium consisting of α -MEM DNA/RNA + 10 vol.% fetal bovine serum + 4 mM L-glutamine + 0.5 vol.% penicillin–streptomycin + 1 vol.% Fungizone® + 100 µM L-ascorbic acid 2-phosphate was used to enhance alkaline phosphatase activity (Declercq et al. 2004).

The cells were cultured for up to 4 months and repeatedly passaged at confluency (approximately every 2 weeks). The morphology was evaluated by phase-contrast microscopy at each passage (P1–P7). When morphological changes were observed, part of the cells were plated into 24-well tissue culture dishes or on Thermanox® coverslips at a concentration of 40,000 cells/well in 0.5 ml osteogenic culture medium (> 6 replicates) to analyze the osteogenic differentiation of the cultures (alkaline phosphatase activity and potential to form a mineralized matrix). The remaining cells were reseeded in T25 tissue culture dishes.

Alkaline phosphatase activity was measured as described below. Once an increase in alkaline phosphatase activity was observed (> 15 mM pNP/mg protein), 10 mM β -glycerophosphate was added to cells cultured in parallel to induce matrix mineralization.

Alkaline phosphatase activity

Alkaline phosphatase activity in the cultures was determined biochemically as well as histochemically.

Biochemical detection

After rinsing the cultures with Ringer solution, the cellular material was removed after lysis into 250 µl of a buffer containing 10 mM Tris–HCl pH 7.5, 0.5 mM MgCl₂ and 0.1% Triton X-100. The cellular material was homogenized by two

freeze-and-thaw cycles. Alkaline phosphatase activity was determined with *p*-nitrophenylphosphate as the substrate. Sample volumes of 50 µl were added to 50 µl *p*-nitrophenylphosphate (4.34 mM) in 100 mM glycine, pH 10.3, 1 mM MgCl₂ and incubated at 37 °C for 30 min on a bench shaker. The enzymatic reaction was stopped by adding 50 µl of 1 M NaOH. Enzyme activity was quantified by absorbance measurements at 405 nm (Universal Microplate Reader EL 800, BIO-TEK instruments, Inc.) and calculated based on a series of *p*-nitrophenol standards (Furuya et al. 1999). Total protein content was determined with the Bradford method from aliquots of the same samples with the Bio-Rad protein assay kit, read at 595 nm (BioPhotometer, Eppendorf) and calculated based on a series of bovine gamma globulin standards. Alkaline phosphatase activity was expressed as mM *p*-nitrophenol/mg protein. Cultures were analyzed in duplicate.

Histochemical staining

After rinsing the cells on the Thermanox® coverslips with cold (4 °C) Ringer solution, the cells were fixed with acetone (– 20 °C) for 5 min. The fixed cultures were washed with cold distilled water and allowed to dry for 30 min. The cultures were incubated for 45 min with Fast Red TR/Naphtol AS-MX or for 10 min with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system at room temperature. The reaction was stopped by removing the substrate solution and washing with distilled water. The Thermanox coverslips were mounted with an aqueous mounting medium or dehydrated in a graded alcohol series and mounted with Canada balsam.

Collagen I immunolocalization

After rinsing the cultures on the Thermanox® coverslips with cold (4 °C) Ringer solution, the cells were fixed with 4% formaldehyde buffered with 10 mM phosphate (pH 6.9) (4 °C, 10 min). The fixed cultures were washed with cold distilled water and let to dry.

The cultures were incubated for 30 min with blocking serum (0.1% BSA in PBS, 0.1% Tween 20® and 0.5% normal swine serum), followed by an incubation (2 h) with rabbit anti rat collagen I

(1/1600 dilution). After washing with PBS, biotinylated swine anti rabbit serum was added, and the cells were incubated for another 30 min. After washing with PBS, the cells were incubated for 30 min with peroxidase-conjugated streptavidin followed by a 10 min incubation period with DAB peroxidase substrate. After washing with tap water and counterstaining with hematoxyline, the Thermanox[®] coverslips were dehydrated in a graded alcohol series and mounted with Canada balsam.

Mineralization of the extracellular matrix

Different methods were used to analyze the mineral deposits in the cultures. The presence of phosphate deposits was analyzed histochemically by Von Kossa staining. The crystalline structure of the calcium phosphate deposits was analyzed qualitatively by transmission electron microscopy and infrared spectrometry.

Phosphate staining (Von Kossa)

Calcium phosphate deposits can be detected by the Von Kossa technique in which phosphate deposits are stained black.

The cultures were rinsed with cold (4 °C) Ringer solution and fixed with 4% formaldehyde buffered with 10 mM phosphate (pH 6.9) (4 °C, 10 min). After washing with cold distilled water, the fixed cultures were covered with a 5% silver nitrate solution and kept for 30 min in a dark room. Afterwards, they were rinsed with distilled water and a sodium-carbonate-formaldehyde solution (5 g Na₂CO₃ in 75 ml distilled water with added 25 ml of 36% formaldehyde) was added for 2 min. The cultures were rinsed in distilled water for 10 min and incubated for 20 min with a staining solution, according to Farmer, containing 1 part of a 10% K₄Fe(CN)₆ · 3H₂O solution and 9 parts of a 10% sodium thiosulphate solution (Böck 1984). The Thermanox[®] coverslips were dehydrated in a graded alcohol series and mounted with Canada balsam.

Transmission electron microscopy

Ultrastructural analysis was carried out to visualize the collagen I fibers and the mineral deposits in the extracellular matrix.

Thermanox[®] monolayers on which three-dimensional nodules were grown, were incubated in a non-enzymatic dissociation reagent while cutting the nodule away from the surrounding monolayer. The nodules were washed with Ringer solution, fixed with 1% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.2) (4 °C, 1 h), and then washed in the same buffer.

The nodules were postfixed with osmium tetroxide, dehydrated in graded concentrations of alcohol and embedded in epoxy-resin. Thin sections (60 nm) were cut with a diamond knife, mounted on copper grids, stained with uranyl acetate and lead citrate and examined using a JEOL 1200 EX II transmission electron microscope operating at 80 KeV.

Infrared spectrometry

The physical and chemical composition of the mineral phase was determined using infrared spectrometry.

The cell layer was rinsed with Ringer solution and lysed and scraped into 250 µl of a buffer containing 10 mM Tris-HCl pH 7.5, 0.5 mM MgCl₂ and 0.1% Triton X-100. After centrifugation at 9000 rpm for 3 min, the supernatant was removed and the cell pellet was dried. FTIR spectra of the samples dispersed in KBr were recorded in the range 400–4000 cm⁻¹ with a resolution of 1 cm⁻¹ using a Mattson Galaxy 6030 FTIR spectrophotometer. The spectra were compared with those of a commercial collagen sample and calcium phosphate standards prepared in our laboratory (Driessens et al. 1990; Pieters et al. 1998).

Results

Cells from the osteogenic layers of the periost were obtained by explant cultures of periosteal fragments. Periost surrounding the tibiae of adult rats (*n* = 12) was used.

Cells migrated from the tissue within 3–7 days after harvest. Confluence near the periosteal explants, cultured in standard medium, was reached within 18 ± 7 days.

For all cell cultures, P0 cells (primary culture) had a fibroblast-like morphology (Figure 1a).

A large variety in the yield of cells was observed between the different experiments. The amount of

Figure 1. Differentiation of periosteum-derived cells. Morphological aspects in extended culture periods (> 4 months). Phase contrast microscopy. a: monolayer of fibroblast-like cells (P0 culture); b, c: little cuboidal cells are observed throughout the culture (P3 culture, 4 days in subculture); d: three-dimensional nodule formation (P3 culture, 14 days in subculture); e: mineralization of a three-dimensional nodule after the addition of β -glycerophosphate (P3 culture, 19 days in subculture); f: unmineralized three-dimensional nodules (P4 culture, 10 days in subculture).

cells from outgrowths of periosteum of the tibiae of 1 adult rat was ranged from 5,00,000 till 4,00,000 cells.

Cells cultured in osteogenic medium and sub-cultured at confluency (approximately every 2 weeks) for up to 7 passages during a period of 3–4 months showed the following characteristics.

The alkaline phosphatase activity of 75% of the cultures ($n = 9$) was low (<5 mM pNP/mg

protein) and did not show any increase during this culture period. Only fibroblast-like cells uniformly distributed over the tissue culture dish were observed.

On the contrary, 25% of the cultures ($n = 3$) (periosteum A, B and C) showed an increasing alkaline phosphatase activity starting from the 2nd or 3rd passage (Figure 2a–c). The activity started from negligible (<5 mM pNP/mg

Figure 2. Alkaline phosphatase activity (mM pNP/mg protein) of periosteum-derived cells (three independent experiments) (periosteum A, B and C) at different passages (P1–P7). Results are the mean and standard deviation of duplicate cultures. (a) Periosteum A was analyzed at P1, P2 and P4. (b) Periosteum B was analyzed at P1, P3, P6 and P7. (c) Periosteum C was analyzed at P1, P2, P3, P4 and P6.

protein) although a slight elevation at P1 or P2, depending on the culture could be observed. The activity increased towards 25 mM pNP/mg

protein, already at the 3rd or 4th passage. The alkaline phosphatase activity increased towards 45 mM PNP/mg protein and remained at

that level even after further subculturing until passage 7.

First or second passage cultures showed cells uniformly distributed over the tissue culture dish with cells merely having a fibroblast-like morphology. As already mentioned, the alkaline phosphatase activity of these cultures is negligible (< 5 mM pNP/mg protein) (Figure 2a–c). Nevertheless, some little cuboidal cells could be observed. Histochemical detection of alkaline phosphatase revealed that these cells were positive and the surrounding fibroblast-like cells were negative (Figure 3a). These cuboidal cells were also positive for collagen I by immunolocalization (Figure 3b).

After further culturing (P3), the confluent monolayer of the periosteum-derived cells showed several spots of little cuboidal cells with fibroblast-like cells around them (Figure 1b, c). Simulta-

neously, the alkaline phosphatase activity increased to 15–40 mM pNP/mg protein (Figure 2b, c).

After several days, the cuboidal spots were present throughout the whole culture. Three-dimensional growth was observed which resulted in several nodular-like structures (Figure 1d). Groups of cells in nodular-like structures showed intense staining for alkaline phosphatase (Figure 4a).

Further subculturing of the cuboidal cells or nodular-like structures (until passages 4–6, depending on the culture) did not influence nodule formation capacity (Figure 1e, f) and alkaline phosphatase activity (Figure 2a–c). Within a few days in subculture, new nodules were formed. The nodules became fully mineralized within 4 days after the addition of β -glycerophosphate (Figure 1e). Mineralization was restricted to the well defined three-dimen-

Figure 3. Periosteum-derived osteoprogenitor cells (P1 culture). (a) Alkaline phosphatase staining (Fast Red) and (b) collagen I immunostaining. Alkaline phosphatase activity and collagen I expression is restricted to the cuboidal cells (light microscopy).

Figure 4. Differentiated periosteum-derived cells (P3 culture, 19 days in subculture). Alkaline phosphatase staining (BCIP) of an unmineralized three-dimensional nodule (a), Von Kossa staining of an unmineralized (b) and mineralized (c) nodule (light microscopy).

sional area and was sharply margined. Von Kossa (Figure 4b, c) staining was positive. The surrounding fibroblast-like cells, which are alkaline phosphatase negative, were not mineralized (Figure 1e).

Ultrastructural analysis by electron microscopy revealed below the upper osteoblast layer numerous, well-banded collagen fibrils as well as needle like electron dense deposits, indicative for the presence of bone apatite crystalline structures (Figure 5a). Vesicular structures, which appear to be the initial sites of mineral deposition, were

present between the collagen fibrils as shown in Figure 5b.

From P5–P6, nodule formation after subculturing decreased or became totally absent. Yet, the alkaline phosphatase activity remained high (50 mM pNP/mg protein). After addition of β -glycerophosphate, alterations in the mineralization pattern were observed. Mineralization not only remained restricted to the three-dimensional area of the nodules (Figure 6a), but mineral deposits were also observed throughout a monolayer of alkaline phosphatase positive cells (Figure 6b).

Figure 5. Ultrastructural pictures of mineralized periosteum-derived cultures. (a) Cuboidal cells are observed next to collagen I fibers and needle like crystals. (b) Vesicular structures are present within a network of collagen I fibers (electron microscopy).

Figure 6. Mineralization pattern of periosteum-derived cells (P6 culture). Nodular (a) and diffuse (b) mineralization (phase contrast microscopy).

Extensive subculturing (P6–P7) still resulted in cultures with high alkaline phosphatase activity (± 45 mM pNP/mg protein) although their nodule formation capacity seemed lost. The mineralization pattern of these extensively subcultured cells was diffuse and spread over large areas throughout the monolayer. Analysis of these diffuse mineral deposits by FTIR demonstrates that the mineral phase is a B-type carbonated apatite (Figure 7a). The asymmetric bending vibration of PO_4^{3-} is split in two distinct peaks at 571 and 601 cm^{-1} and the PO_4^{3-} absorption band in the region 950 – 1300 cm^{-1} is comparable to that of the apatite spectrum in Figure 7b next to the typical protein absorption peaks. In addition, not only the bending vibration of CO_3^{2-} at 872 cm^{-1} is clearly seen, but also the stretching vibration at 1420 cm^{-1} corroborating that the mineral phase is a B-type carbonated apatite in which PO_4^{3-} ions are substituted by CO_3^{2-} ions comparable to bone mineral (Driessens et al. 1990; Pieters et al. 1998).

Figure 7. FTIR-spectrum of cell pellets of mineralized periosteum-derived cells (a) in comparison with the spectrum of carbonate-containing apatite (b).

Cells subcultured from P7–P8 failed to adhere and were dying off.

Discussion

In bone fracture healing, it is important that restoration of the damaged area results in the formation of new bone, having the same integrity as the surrounding bone tissue. For this purpose, materials are designed that should attract appropriate cells which become bone forming cells at the cell/biomaterial interface and replacing in time the degradable biomaterial by newly formed bone. Cells from the osteogenic bone lining layers of the periosteum are the first contributors to the regeneration of damaged bone tissue. Therefore, it could be valuable to use these specific progenitor cells in culture models to study the osteoconductive and -inductive capacities of biomaterials, developed for bone regenerative therapy.

In the present paper, separate cultures were started, using the periosteum of both tibiae from each adult rat ($n = 12$). Cell cultures were obtained by migration of cells from periosteal fragments (explant culture) and monolayer-expanded. In the literature, explant culture (Koshihara et al. 1987, 1989; Bahrami et al. 2000; Hanada et al. 2001) as well as enzymatic digestion (Iwasaki et al. 1994; Solchaga et al. 1998; De Bari et al. 2001; Gruber et al. 2001) of periosteal fragments are described to isolate periosteal cells, from a diversity of animals and human. However, in our opinion, explant cultures have a higher probability to result in cultures with osteogenic potential. Isolation of cells by enzymatic treatment of periosteal fragments not

only liberates cells from the osteogenic lineage (deriving from the cambium layer), but also non-osteogenic fibroblasts (deriving from the fibrous layer). The periosteum present around the long bones of rats consists of a thin layer, from which it is impossible to separate the osteogenic layer from the fibrous layer prior to enzymatic digestion. Moreover, by using periosteal tissue fragments instead of cell suspensions to start the cultures, the outgrowing cells are derived from an intact tissue (layer), a situation more similar to that *in vivo* when damaged bone has to be repaired. For the above mentioned reasons, we preferred working with periosteal explants instead of enzymatically released cells. The osteogenic layer was directed towards the bottom of the culture dish, allowing attachment and expansion of the cells from the cambium layer and minimizing contamination with fibroblasts from the upper fibrous layer.

Despite the use of explant-expanded cells, 75% of the cultures ($n = 9$) did not show any increase in alkaline phosphatase activity nor nodule formation during long-term culture for up to 4 months. Morphology was dominated by spindle-shaped, fibroblast-like cells, suggesting that contamination of the cultures with cells derived from the fibrous layer of the periosteum was the reason for the above mentioned observations. Difficulties to harvest the mesenchymal cells from the cambium layer are often described. This cambium layer has a tendency to remain attached to the underlying bone when the explant is taken (Breitbart et al. 1998). Nevertheless, even when the cambium layer together with the fibrous layer was harvested successfully, contamination with fibroblasts is feasible. These contaminant non-progenitor cells can inhibit the differentiation of the mesenchymal cells in the cultures (Iwasaki et al. 1995; Solchaga et al. 1998).

However, in other cultures, an elevation of alkaline phosphatase activity was already observed from the first or second passage. Simultaneously with the increase in alkaline phosphatase activity, populations of round cuboidal cells appeared in the culture. Over time, alkaline phosphatase activity was still increasing and three-dimensional nodule formation was observed. The presence of two morphological distinct types of cells in periosteal-derived cultures at early passages was also described by others. The round cuboidal cells are considered to be the osteo/chondroprogenitor cells

(Izumi et al. 1992; Hanada et al. 2001). According to Izumi et al. (1992), alkaline phosphatase expression in the periosteum is not from fibroblasts or chondrocyte precursors, but from osteoblasts and osteoblast precursors. In our culture system, the simultaneous appearance of these cuboidal cells and the increase in alkaline phosphatase activity, with the subsequent formation of three-dimensional nodules able to mineralize in an osteogenic medium, supports these findings.

With time in culture, these cuboidal cells capable of nodule formation are present throughout the culture. This suggests that the culture is enriched in alkaline phosphatase-positive progenitor cells or assumes that almost all the cells are able to differentiate into osteoblasts. The latter was described by Hanada et al. (2001), 2 types of cells, alkaline phosphatase-positive and alkaline phosphatase-negative cells are present at early culture periods. These alkaline phosphatase-negative cells are not only contaminating fibroblasts from the outer layer but also undifferentiated mesenchymal progenitor cells capable of differentiating into the osteogenic lineage.

Extensive subculturing (up to P7) resulted in cultures with altered mineralization patterns. Early subcultures showed a mineralization pattern restricted to nodular structures. Extensive subculturing showed dispersed mineralization all over the cultures. The mineralization patterns observed in the latter are comparable with the patterns observed in cultures derived from rat osteosarcoma (UMR-106) and in osteoblast cultures derived from long bones (Declercq et al. 2004).

Compared to osteoblast cultures derived from long bones and calvaria from adult or fetal rat (Declercq et al. 2004), periosteum-derived cultures have the advantage of prolonged cultivation before terminal differentiation and the consequent dying off of the cultures. Osteoblastic cells at a higher differentiation stage (osteoblast cells and calvaria cells) have a high alkaline phosphatase activity in first passage cultures (>10 mM pNP/mg protein) and have the ability to differentiate to their end-phase within 21 days whereas periosteum-derived cells have a negligible alkaline phosphatase activity at first or second passage cultures. These less differentiated cells can be cultured without losing the potential of osteogenesis. As a consequence, the cells can be expanded for up to 7 passages before terminal osteoblastic differentiation.

In conclusion, we want to emphasize the importance of periosteum-derived cell cultures to study biomaterial/cell interactions. Despite the difficulties to obtain the correct material (periosteum with an intact osteogenic layer) and despite the fact that a lot of cultures were not capable of being induced to become differentiated osteoblast cultures, periosteum-derived cultures have several advantages compared to bone derived cultures. First, there is the biological relevance of the periosteal cells being the first contributors to repair damaged bone. Secondly, their osteogenic potential persists even after prolonged cultivation. Longer culture periods (3–4 months) can be performed before final differentiation is obtained, making these cultures suitable for long-term *in vitro* evaluation of biomaterials (Perka et al. 2000; Arnold et al. 2002). A point to note here is that selection of the appropriate cultures having an osteogenic potential is a necessity. Working with non-inducible cultures would result in wrong conclusions about the osteoconductive and -inductive capacities of biomaterials. This selection can be done at an early time point during the *in vitro* culturing, by selecting those cultures showing at early passages (P1–P2) the appearance of cuboidal cells. However, the isolation and selection of these cultures having an osteogenic capacity is time consuming. Hence, a more appropriate source to study cell/biomaterial interactions should be more convenient e.g. the use of bone marrow derived cells (Declercq et al. 2005).

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References

- Arnold U., Lindenhayn K. and Perka C. 2002. In vitro-cultivation of human periosteum derived cells in bioresorbable polymer-TCP-composites. *Biomaterials* 23: 2303–2310.
- Attawia M.A., Devin J.E. and Laurencin C.T. 1995. Immunofluorescence and confocal laser scanning microscopy studies of osteoblast growth and phenotypic expression in three-dimensional degradable matrices. *J. Biomed. Mater. Res.* 29: 843–848.
- Bahrami S., Stratmann U., Wiesmann H.P., Mokrys K., Bruckner P. and Szuwart T. 2000. Periosteally derived osteoblast-like cells differentiate into chondrocytes in suspension culture in agarose. *Anat. Rec.* 259(2): 124–130.
- Böck P. 1984. *Der Semidünnschnitt*. J.F. Bergmann Verlag, München, pp. 98–99.
- Breitbart A.S., Grande D.A., Kessler R., Ryaby J.T., Fitzsimmons R.J. and Grant R.T. 1998. Tissue engineered bone repair of calvarial defects using cultured periosteal cells. *Plast. Reconstr. Surg.* 101: 567–576.
- Burdick J.A., Padera R.F., Huang J.V. and Anseth K.S. 2002. An investigation of the cytotoxicity and histocompatibility of *in situ* forming lactic acid based orthopedic biomaterials. *J. Biomed. Mater. Res. (Appl. Biomater.)* 63: 484–491.
- Calandrelli L., Immirzi B., Malinconico M., Orsello G., Volpe M.G., Della Ragione F. and Zappia V. 2002. Biocompatibility studies on biodegradable polyester-based composites of human osteoblasts: a preliminary screening. *J. Biomed. Mater. Res.* 59: 611–617.
- Dalby M.J., Di Silvio L., Harper E.J. and Bonfield W. 2002. Increasing hydroxyapatite incorporation into poly(methylmethacrylate) cement increases osteoblast adhesion and response. *Biomaterials* 23: 569–576.
- De Bari C., Dell'Accio F. and Luyten F.P. 2001. Human periosteum-derived cells maintain phenotypic stability and chondrogenic potential throughout expansion regardless of donor age. *Arthritis Rheum.* 44(1): 85–95.
- Declercq H., Van den Vreken N., De Maeyer E., Verbeeck R., Schacht E., De Ridder L. and Cornelissen R. 2004. Isolation, proliferation and differentiation of osteoblastic cells to study cell/biomaterial interactions: comparison of different isolation techniques and source. *Biomaterials* 25: 757–768.
- Declercq H.A., Verbeeck R.M.H., De Ridder L.I.F.J.M., Schacht E.H. and Cornelissen M.J. 2005. Calcification as an indicator of osteoinductive capacity of biomaterials in osteoblastic cell cultures. *Biomaterials* 26: 4964–4974.
- Driessens F.C.M. and Verbeeck R.M.H. 1990. *Biominerals*. CRC Press, Boca Raton.
- Furuya K., Nifuji A., Rosen V. and Noda M. 1999. Effects of GDF7/BMP12 on proliferation and alkaline phosphatase expression in rat osteoblastic osteosarcoma ROS 17/2.8 cells. *J. Cell Biochem.* 72: 177–180.
- Gruber R., Mayer C., Bobacz K., Krauth M., Graninger W., Luyten F.P. and Erlacher L. 2001. Effects of cartilage-derived morphogenetic proteins and osteogenic protein-1 on osteochondrogenic differentiation of periosteum-derived cells. *Endocrinology* 142: 2087–2094.
- Hanada K., Solchaga L.A., Caplan A.I., Hering T.M., Goldberg V.M., Yoo J.U. and Johnstone B. 2001. BMP-2 induction and TGF-beta1 modulation of rat periosteal cell chondrogenesis. *J. Cell Biochem.* 81: 284–294.
- Ishaug S.L., Crane G.M., Miller M.J., Yasko A.W., Yasemski M.J. and Mikos A.G. 1997. Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. *J. Biomed. Mater. Res.* 37: 17–28.
- Iwasaki M., Nakahara H., Nakase T., Kimura T., Takaoka K., Caplan A.I. and Ono K. 1994. Bone morphogenetic protein 2 stimulates osteogenesis but does not affect chondrogenesis in osteochondrogenic differentiation of periosteum-derived cells. *J. Bone Miner. Res.* 9(8): 1195–1204.

- Iwasaki M., Nakahara H., Nakata K., Nakase T., Kimura T. and Ono K. 1995. Regulation of proliferation and osteochondrogenic differentiation of periosteum-derived cells by transforming growth factor- β and basic fibroblast growth factor. *J. Bone Joint Surg.* 77-A(4): 543–554.
- Izumi T., Scully S.P., Heydemann A. and Bolander M.E. 1992. Transforming growth factor β 1 stimulates type II collagen expression in cultured periosteum-derived cells. *J. Bone Miner. Res.* 7(1): 115–121.
- Koshihara Y., Kawamura M., Endo S., Tsutsumi C., Kodama H., Oda H. and Higaki S. 1989. Establishment of human osteoblastic cells derived from periosteum in culture. *In Vitro Cell Dev. Biol.* 25(1): 37–43.
- Koshihara Y., Kawamura M., Oda H. and Higaki S. 1987. In vitro calcification in human osteoblastic cell line derived from periosteum. *Biochem. Biophys. Res. Commun.* 145(2): 651–657.
- Lisignoli G., Zino N., Remiddi G., Piacentini A., Puggioli A., Trimarchi C., Fini M., Maraldi N.M. and Facchini A. 2001. Basic fibroblast growth factor enhances in vitro mineralization of rat bone marrow stromal cells grown on non-woven hyaluronic acid based polymer scaffold. *Biomaterials* 22: 2095–2105.
- Moursi A.M., Winnard A.V., Lannutti J.J. and Seghi R. 2002. Enhanced osteoblast response to a polymethylmethacrylate-hydroxyapatite composite. *Biomaterials* 23: 133–144.
- Perka C., Schultz O., Spitzer R., Lindenhayn K., Burmester G. and Sittinger M. 2000. Segmental bone repair by tissue-engineered periosteal cell transplants with bioresorbable fleece and fibrin scaffolds in rabbits. *Biomaterials* 21: 1145–1153.
- Pieters I.Y., De Maeyer E.A.P. and Verbeeck R.M.H. 1998. Influence of Na^+ on the stoichiometry of carbonated hydroxyapatite obtained by the hydrolysis of octacalcium phosphate. *Inorg. Chem.* 37: 6392–6395.
- Salgado A.J., Coutinho O.P. and Reis R.L. 2004. Bone tissue engineering: state of the art and future trends. *Macromol. Biosci.* 4: 743–765.
- Schwartz E. 1997. Culture of specific cell types, mesenchymal cells, bone. In: Freshney R.I. (ed.), *Culture of Animal Cells. A Manual of Basic Technique*, 3rd ed. Wiley-Liss, Inc., New York, pp. 332–333.
- Scotchford C.A., Cascone M.G., Downes S. and Giusti P. 1998. Osteoblast responses to collagen-PVA bioartificial polymers in vitro: the effects of cross-linking method and collagen content. *Biomaterials* 19: 1–11.
- Solchaga L.A., Cassiède P. and Caplan A.I. 1998. Different response to osteo-inductive agents in bone-marrow and periosteum-derived cell preparations. *Acta Orthop. Scand.* 69(4): 426–432.
- Spitzer R.S., Perka C., Lindenhayn K. and Zippel H. 2002. Matrix engineering for osteogenic differentiation of rabbit periosteal cells using α -tricalcium phosphate particles in a three-dimensional fibrin culture. *J. Biomed. Mater. Res.* 59: 690–696.