Bone Augmentation With Autologous Periosteal Cells and Two Different Calcium Phosphate Scaffolds Under an Occlusive Titanium Barrier: An Experimental Study in Rabbits

Marina Maréchal,*† Jeroen Eyckmans,‡ Jan Schrooten,§ Evert Schepers,‖ Frank P. Luyten,‡¶ and Daniel van Steenberghe*

Background: This study used a tissue-engineering approach, which combined autologous periosteal cells with a scaffold material, to promote bone augmentation under an occlusive titanium barrier that was placed on the skull of rabbits. Because the cell–matrix interaction is of key importance in tissue engineering, two different calcium phosphate–based scaffolds were seeded with autologous periosteal cells. One scaffold contained hydroxyapatite, tricalcium phosphate, and collagen; the other scaffold was a β-tricalcium phosphate structure.

Methods: The experiment involved 38 rabbits divided into five groups: the two different scaffolds with and without cells and a blood clot only. Prior to implantation, autologous periosteal cells were harvested from the tibia by stripping the periosteum. Cells were cultured, and 1 day before the implantation ~20 million cells were collected and seeded onto the scaffolds. Two preformed dome–shaped full titanium barriers were placed subperiosteally onto the frontal and parietal bones of each rabbit. Before placement of the barriers, the different scaffolds, seeded with or without cells, were put on top of the skull. As a negative control, autologous blood was injected into the barriers. Histologic evaluation and histomorphometric analysis were performed after 12 weeks of undisturbed bone growth. Measurements involved the amounts of newly formed tissue and of new bone distinguishing between trabecular bone and osteoid.

Results: No significant differences were found between the four treatment groups (scaffolds with or without cells). However, the amount of new bone tissue found underneath the titanium barriers with scaffolds was significantly higher ($P<0.04$) than with a blood clot only.

Conclusion: A better understanding of the mode of action is required to optimize tissue-engineering procedures before entering clinical applications. *J Periodontol 2008;79:896-904.*

KEY WORDS

Animal studies; barriers; bone and bones; cells; tissue engineering; tissue scaffolds; tricalcium phosphate.

* Department of Periodontology, Catholic University of Leuven, Leuven, Belgium.
† TiGenix, Heverlee, Belgium.
‡ Department of Musculoskeletal Sciences, Laboratory for Skeletal Development and Joint Disorders, Catholic University of Leuven.
§ Department of Metallurgy and Materials, Catholic University of Leuven.
‖ Department of Prosthetic Dentistry, Catholic University of Leuven.
¶ Department of Musculoskeletal Sciences, Catholic University of Leuven.

For decades, the regeneration of damaged or lost jaw bone tissue has been one of the major endeavors in periodontal and maxillofacial surgery. Defects of the jaw bone may be congenital or due to involution (e.g., after tooth loss), pathology, trauma, or tumor resections. Periodontists and craniofacial surgeons often resolve these esthetic and functional disorders by inserting implants to retain or support a dental or nasal prosthesis, despite an insufficient amount of bone. A limited jaw bone volume can be increased by an onlay graft. This can be harvested from the iliac crest and is usually a horseshoe-shaped bone graft.[1,2] It is still the gold standard because of its osteoconductive and osteoinductive features and limited resorption over the years if properly stimulated by endosseous implants. Limitations involve

doi: 10.1902/jop.2008.070043

the amount of available bone, the requirement of a second surgical site, and the morbidity of the donor site.

An alternative approach is the use of allografts, such as demineralized freeze-dried bone allograft. Unfortunately, they have unpredictable osteoinductive properties.[3] There is also some concern of a possible transfer of pathogenic factors, such as prions.[4] Therefore, xenogenic grafts (grafts shared between different species, such as bovine porous bone mineral or a natural coral) offer a reassuring alternative because such risk can be eliminated more easily, although perhaps not completely. These materials are biocompatible and osteoconductive.[5] Besides these xenogenic grafts, the use of a large variety of synthetic bone substitutes can also be envisioned. However, using alternative materials, like hydroxyapatite (HA) or tricalcium phosphate (TCP), only promotes osteoconduction, which is more rapid for HA than for TCP.[6] Conversely, the resorption of HA is much slower than for TCP particles.[7]

An alternative approach for bone augmentation consists of the placement of space-maintaining subperiosteal barrier membranes with or without bone substitutes underneath. Using expanded Teflon membranes reinforced by titanium, a vertical jaw bone augmentation of 4 mm can be achieved over limited segments.[8] Larger gains in vertical bone height (up to 16 mm) can be obtained over entire maxillae by means of a preformed, custom-fit, fully occlusive titanium barrier.[9] Some drawbacks of the latter approach are known, such as the long healing time (>1 year) and the low degree of mineralization of the newly formed bone.[9]

The aim of the present experiment was to evaluate the contribution of tissue-engineering techniques, i.e., the use of a combination of a suitable scaffold with osteogenic cells.

The experiment was designed to evaluate the effect of periosteal cells on bone augmentation. Cells derived from the periosteum are capable of inducing bone formation. The first in vivo experiments to evaluate the bone-forming capacity of periosteum-derived cells were performed in nude mice. Periosteal cells isolated from young chickens were subcutaneously implanted in nude mice.[10] Such cultured cells inoculated into nude mice gave rise to new bone tissue in this subcutaneous injection site. When these cells were loaded into diffusion chambers and implanted intraperitoneally, they also led to bone formation.[11] The potential of these periosteum-derived cells was also demonstrated in other species.[12] The same osteogenic capacity of periosteal cells was documented in rabbits; cells were brought in a defect created in the tibia through distraction osteogenesis.[13] Recently, the mesenchymal multipotency of adult human periosteal cells was demonstrated by single-cell lineage analysis.[14]

The clinical application of these cultured cell populations to heal large bone defects seems to be the next step, but it is still in an experimental phase. Vacanti et al.[15] were the first to publish a clinical application of periosteal cells in bone-tissue engineering in a phalanx reconstruction. They made use of 20 million periosteal cells embedded into a calcium phosphate scaffold.

A porous bioceramic scaffold made of HA and/or TCP was chosen for the present study to anchor the periosteal cells. The presence of a mineralized scaffold microenvironment is a requirement for the onset of bone formation by committed human osteogenic cells.[16] The internal porosity favors bone deposition by osteogenic cells and may also facilitate scaffold resorption. Adding a high concentration of TCP to the bioceramic usually results in faster scaffold resorption; two scaffold compositions were used. To evaluate the possible difference in bone formation and resorption of the scaffold over time, one scaffold (HA/TCP)# was made of HA, TCP, and highly purified type 1 bovine dermal fibrillar collagen; the other scaffold (TCP)** was a pure β-TCP structure.

MATERIALS AND METHODS

Thirty-eight New Zealand white rabbits, with an average weight of 2.6 kg and aged 11 weeks, were used in this study. All animals were housed individually in standard cages and had access to water and food *ad libitum*. The experimental protocol was approved by the Animal Experiments Committee of the Catholic University of Leuven.

The Harvest of Periosteal Tissue

During the first surgery, a piece of periosteum was stripped from the tibia of the rabbits to perform periosteal cell isolation and culture.

Thirty minutes before surgery, the rabbits were given analgesics†† and an antibiotic‡‡ intramuscularly. Surgery was performed under systemic anesthesia, with ketamine§§ and xylazine‖‖ injected intramuscularly.

Under sterile conditions, an incision was made at the medial side of the right proximal tibia. A 5 × 15-mm piece of periosteum was harvested from the tibia by stripping. The periosteal specimen was put into a high-glucose Dulbecco's modified Eagle's medium (DMEM)¶¶ supplemented with 10% fetal bovine serum

Collagraft, Neucoll, Campbell, CA.
** Vitoss, Orthovita, Malvern, PA.
†† Buprenorphine, 0.03 mg/kg, Temgesic, Schering-Plough, Brussels, Belgium.
‡‡ Benzylpenicillum, 0.25 ml/kg, Continental Pharma, Brussels, Belgium.
§§ Ketamine 1000, 30 mg/kg, Ceva Sante Animale, Brussels, Belgium.
‖‖ Xyl-M, 5 mg/kg, VMD, Arendonk, Belgium.
¶¶ Life Technologies, Merelbeke, Belgium.

(FBS)[##] and 1× antibiotic-antimycotic solution[***] as a transport medium. The skin incision was closed carefully with bioabsorbable sutures.[†††] Postoperatively, the rabbits received analgesics[‡‡‡] twice daily for 2 days and antibiotics once a day for 3 days.[§§§]

Cell Isolation, Cell Culture, and Cryopreservation

Within 1 hour after harvesting, the periosteal specimens were transported to the laboratory, finely minced, and digested overnight with 0.2% crude type IV collagenase[‖‖‖] in high-glucose DMEM containing 10% FBS and 1× antibiotic-antimycotic solution. Following overnight incubation at 37°C, periosteal cells were collected by centrifugation. After removal of the supernatant, the cells were resuspended in growth medium (DMEM supplemented with 10% FBS and 1× antibiotic-antimycotic solution), plated in a T25 culture flask, and allowed to attach for 5 days. Non-adherent cells were removed by changing the medium.

For expansion, cells were cultured in monolayer in growth medium at 37°C in a humidified atmosphere of 5% CO_2. After 6 to 8 days, the periosteum-derived cells reached confluence; they were washed twice with calcium- and magnesium-free phosphate buffered saline,[¶¶¶] released from the plastic by treatment with trypsin-EDTA,[###] and replated at 1:6 dilution for the first subculture. Cell passages were continued with a cell-seeding density varying between 10,000 and 15,000 cells/cm^2. After two passages, the cells were suspended in DMEM with 20% FBS and 10% dimethylsulfoxide,[****] to prevent the formation of ice crystals, which will break down the cell membrane during cryopreservation, and cryopreserved in liquid nitrogen.

Cell Seeding

The cells were thawed and put into culture 7 days before the second surgery. The cells were cultured in a growth medium (DMEM supplemented with 10% FBS and 1× antibiotic-antimycotic solution).

After 6 days, 20 million viable cells were collected to be seeded onto two different scaffolds (HA/TCP[††††] and TCP[‡‡‡‡]) on the day before surgery. HA/TCP is made of 65% HA, 35% TCP, and highly purified type 1 bovine dermal fibrillar collagen. HA/TCP has excellent biocompatibility and closely resembles natural bone (Fig. 1). TCP is a pure β-TCP, a natural material containing 39% calcium and 20% phosphorus. Eight percent to 12% by volume correlates to 88% to 92% interconnected porosity, and pore sizes range from <1 to 1,000 μm in diameter (Fig. 1). HA/TCP was cut into blocks of ~5 × 5 × 5 mm because that size is well documented for cell-seeding procedures in our laboratory. TCP was cut into blocks of ~10 × 8 × 6 mm (length, width, and height, respectively). Because TCP was too brittle to be cut in smaller pieces of similar size as the HA/TCP, differences in geometry were accepted.

Figure 1.
TCP (left) and HA/TCP (right) as received from manufacturers.

These blocks were dropped into 150 μl cell suspension. After overnight incubation at 37°C, the seeded scaffolds were put into an Eppendorf tube with 1 ml serum-free medium 1.5 hours before implantation.

After implantation, the non-attached cells in the supernatant were counted to estimate the seeding efficiency, which was calculated as follows: ([number of seeded cells – number of cells in the supernatant]/number of seeded cells) × 100. The viability of the cells in the supernatant was assessed by trypan blue exclusion test. Data were expressed as an average, and the statistical significance was determined using the Mann-Whitney U test to compare averages between the groups, with a P value <0.01 being considered significant.

Titanium Barriers

Dome-shaped commercially pure titanium barriers were molded by hydroforming from a 0.2-mm-thick titanium foil. The maximum available bone surface was used to design two cups (Fig. 2) along the anteroposterior axis, allowing slightly greater dimensions for the posterior cup. The titanium barriers were cleaned in a series of alcohol dilutions in an ultrasonic bath before autoclaving.

BioWhittaker, Verviers, Belgium.
*** 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B, Life Technologies.
††† Vicryl Rapide, Johnson & Johnson, Brussels, Belgium.
‡‡‡ Buprenorphine, 0.03 mg/kg, Temgesic, Schering-Plough.
§§§ Benzylpenicillum, 0.25 ml/kg, Continental Pharma.
‖‖‖ Life Technologies.
¶¶¶ BioWhittaker.
0.25% trypsin, 1 mM EDTA, Life Technologies.
**** Sigma, Bornem, Belgium.
†††† Collagraft, Neucoll.
‡‡‡‡ Vitoss, Orthovita.

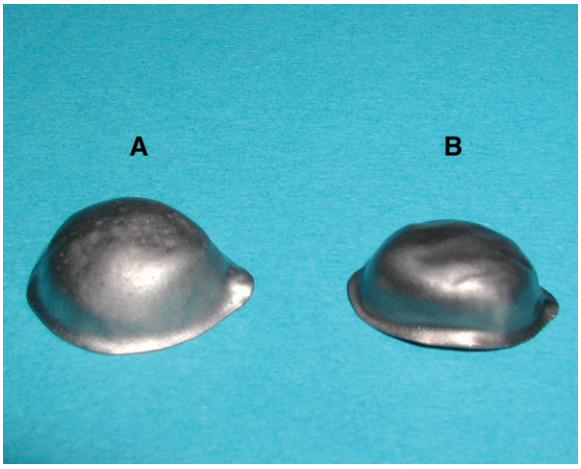

Figure 2.
The posterior cup ***(A)*** *and the anterior titanium cup* ***(B)****.*

Placement of Titanium Cups and Application of the Different Treatment Methods

The titanium barriers were installed on top of the rabbit's skull 6 weeks after the first surgery, and the treatment methods were chosen randomly.

The thirty-eight rabbits were divided into five subgroups according to the treatments: TCP with cells (eight rabbits), HA/TCP with cells (eight rabbits), TCP without cells (eight rabbits), HA/TCP without cells (eight rabbits), and a blood clot only (six rabbits).

An analgesic§§§§ and an antibiotic‖‖‖‖ were given intramuscularly 30 minutes before anesthesia was performed. The surgery at the skull was performed under systemic anesthesia¶¶¶¶#### injected intramuscularly.

Surgery took place under aseptic conditions. A U-shaped cutaneo-periosteal flap was prepared. Titanium barriers were placed: one on the frontal bone and one on the parietal bone. Prior to the placement of the cups, 15 standardized trepanations of the cortical bone were performed with a small round burr to enhance the capillary invasion.[17] The scaffolds with or without cells were placed on top of the skull and covered with a titanium barrier, after which the borders of the cups were glued to the skull with cyanoacrylate glue.***** As a negative control, titanium barriers with the application of a blood clot were used. Blood was collected from the ear artery and injected into the barrier. When the blood clot was coagulated, the barriers with the blood clot were placed on top of the skull and sealed immediately with cyanoacrylate glue.††††† The periosteum and the skin were closed with bioabsorbable sutures.‡‡‡‡‡

Postoperatively, the rabbits received analgesics§§§§§ twice a day for 2 days and antibiotics once a day for 3 days.‖‖‖‖‖ They could eat and drink *ad libitum*. One rabbit died as a result of diarrhea 1 day postoperatively and was withdrawn from the study.

A period of 12 weeks of undisturbed bone growth underneath the titanium cups was chosen because it corresponds to ~1 year in humans.[9] The rabbits were anesthetized with ketamine¶¶¶¶¶ and xylazine##### and sacrificed by means of an intracardial injection of a veterinary euthanasia drug containing embutramide, mebenzoniumiodide, and tetracaine hydrochloride.****** The frontal and parietal bones were removed *en bloc* and then separated. Each titanium barrier was put into a solution of formaldehyde.

Histology and Histomorphometric Analysis

After 2 days of fixation, the blocks were dehydrated in a graded series of ethanol and embedded in methylmetacrylate. Undecalcified serial sections were prepared in a sawing microtome.†††††† The sections were stained with Stevenel's blue and counterstained with van Gieson's picrofuchsin. A histomorphometric analysis was performed with a computerized planimetric program.‡‡‡‡‡‡ Only one section of three was analyzed, as determined in a pilot study.

First, the total volume under the barrier was measured by drawing a line just under the titanium barrier and along the skull by discriminating newly formed bone from the original bone. The total area of the following four parameters was measured: new tissue (bone and fibrous tissue), new bone tissue, newly formed trabecular bone, and osteoid tissue. The areas of the selected sections were expressed in square millimeters, and serial sections were added (10 for the anterior cup and 13 for the posterior cup). The total surface for all four parameters under the barrier was derived from the sum of serial histologic cuts. Subsequently, the volume of all tissues, with whatever characteristics, was calculated as a percentage of the total volume available under the barrier.

For a statistical analysis of the results, the Kruskal-Wallis test was used to compare the percentages of volume occupied by the tissues under the barrier, between the different treatment methods. The Mann-Whitney U test was performed to verify if there was an effect of the scaffold and an effect of adding cells. Mann-Whitney U tests were used to verify whether there was a difference between the four groups, with a scaffold, and the control group (blood clot).

§§§§ Buprenorphine, 0.03 mg/kg, Temgesic, Schering-Plough.
‖‖‖‖ Benzylpenicillum, 0.25 ml/kg, Continental Pharma.
¶¶¶¶ Ketamine 1000, 30 mg/kg, Ceva Sante Animale.
Xyl-M, 5 mg/kg, VMD.
***** Histoacryl, Braun, Melsungen, Germany.
†††††† Histoacryl, Braun.
‡‡‡‡‡ Vicryl Rapide, Johnson & Johnson.
§§§§§ Buprenorphine, 0.03 mg/kg, Temgesic, Schering-Plough.
‖‖‖‖‖ Benzylpenicillum, 0.25 ml/kg, Continental Pharma.
¶¶¶¶¶ Ketamine 1000, 30 mg/kg, Ceva Sante Animale.
Xyl-M, 5 mg/kg, VMD.
****** T61, Hoechst Roussel Vet, Brussels, Belgium.
†††††† Leica SP 1600, Nussloch, Germany.
‡‡‡‡‡‡ Image Pro-Plus, Media Cybernetics, Silver Spring, MD.

RESULTS

Analyzing all barriers, a tissue filling from 80% to 100% was found underneath the titanium barriers, with no significant differences between the anterior and posterior barriers, among the scaffold groups, or between the scaffold groups and the control group. The bone augmentation observed was characterized by slender trabeculae associated with osteoblasts lining the osteoid layer. There was always new bone in close contact with the original skull bone. Bone growth along the inner surface of the titanium barrier, indicating osteoconductivity of the titanium, was negligible. Bone growth was rather initiated from the skull bone and was sometimes combined with a striking resorption of the scaffold. This was mainly observed for the TCP scaffold (Fig. 3). The bone growth in the HA/TCP scaffolds occurred more often in between the particles of the scaffold, with very little resorption of the HA/TCP at the skull base (Fig. 4). A close contact between the scaffold material and the new trabecular bone was observed in both scaffolds (Fig. 5). Resorption of the material was still ongoing based on the presence of some giant cells in between the particles of the scaffold.

A seroma was detected underneath 73% of the titanium barriers. The outline of these seromas consisted of osteoid tissue in which some small bone particles could be detected. In general, these seromas were situated in the empty space between the scaffold and the titanium barriers (Fig. 6). According to Data Sciences International (St. Paul, Minnesota), a seroma is defined as a sterile accumulation of serum in a circumscribed dead space in the tissue.[18] It can be the result of a tissue injury, inflammation, or a body defense mechanism. No detectable cells or fibers were present in the center of the seroma.

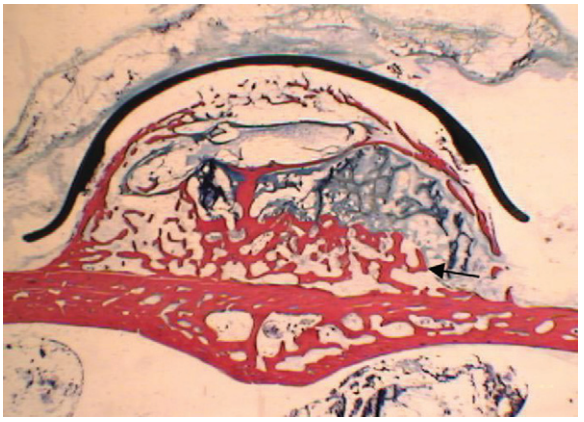

Figure 3.
An anterior cup filled with a TCP scaffold. Ingrowth of new tissue from the skull can be detected together with resorption of the scaffold material in contact with the tabula externa (arrow) (Stevenel's blue and van Gieson's picrofuchsin; original magnification ×6.)

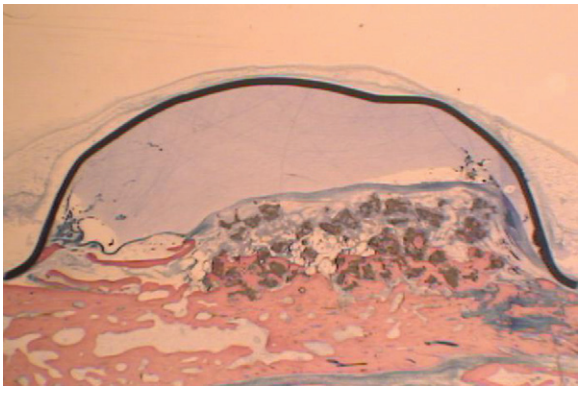

Figure 4.
The new trabecular bone growing in between the particles of HA/TCP under a titanium barrier (Stevenel's blue and van Gieson's picrofuchsin; original magnification ×6.)

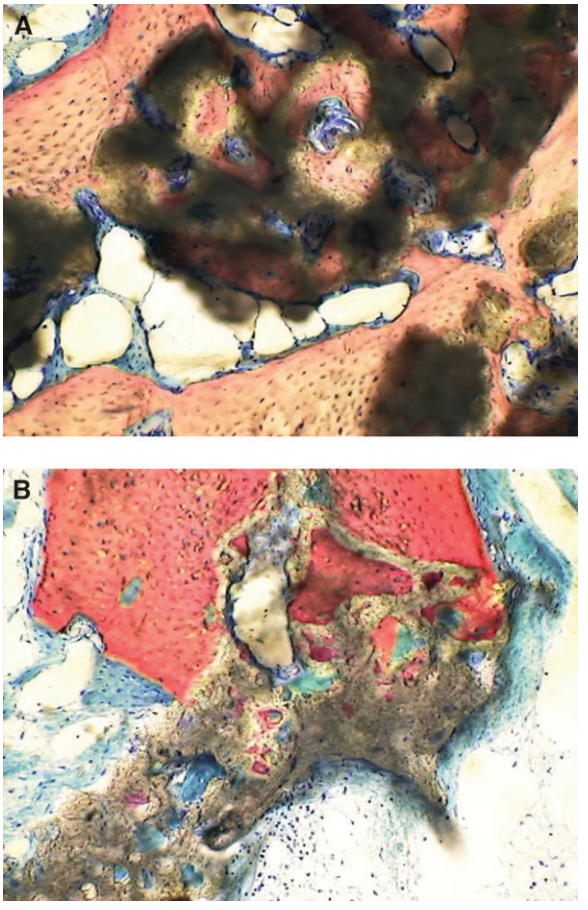

Figure 5.
A) *Close contact between the new bone and the particles of the HA/TCP scaffold.* ***B)*** *TCP scaffold with new trabecular bone and osteoid tissue (green) and fibrous tissue (blue). (Stevenel's blue and van Gieson's picrofuchsin; original magnification ×100.)*

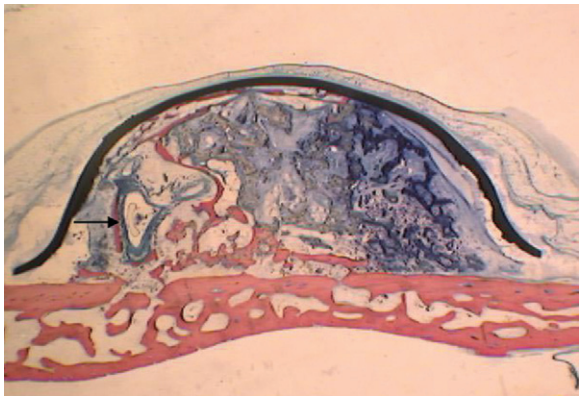

Figure 6.
A seroma (arrow) situated outside a TCP scaffold (Stevenel's blue and van Gieson's picrofuchsin; original magnification ×6.)

Table 1.

Percentages (mean ± SD) of New Tissue, New Bone Tissue, and New Trabecular Bone Under the Titanium Cups

Group	Average percentages (anterior and posterior barrier)
New tissue (bone and fibrous)	
1	97.1 ± 3.2
2	95.2 ± 3.8
3	94.7 ± 8.1
4	96.6 ± 3.6
5	96.0 ± 4.8
New bone tissue	
1	27.8 ± 10.5
2	28.5 ± 10.5
3	32.0 ± 12.3
4	35.4 ± 16.0
5	24.1 ± 7.4
New trabecular bone	
1	7.8 ± 2.0
2	6.9 ± 2.7
3	8.1 ± 2.7
4	8.8 ± 4.0
5	7.0 ± 1.7

Group 1 = TCP and cells, group 2 = HA/TCP and cells, group 3 = TCP without cells, group 4 = HA/TCP without cells, and group 5 = blood clot only.

For all treatment procedures, the variability of the bone growth under the barriers was very high among the animals of each group.

Cell Seeding and Cell Viability After Seeding

Twenty million viable periosteal cells were used for seeding of the two scaffolds. After seeding, the non-attached cells were counted in the supernatant to estimate the seeding efficiency. Cell viability of the periosteum-derived cells in the supernatant was tested by trypan blue exclusion test.

There was a significant difference (P <0.001) between the seeding efficiency on the TCP scaffolds (65.7% ± 7.0%) and the HA/TCP scaffolds (35.8% ± 7.0%).

No significant difference could be detected between the cell viability on the HA/TCP scaffold (60.4% ± 7.0%) and the TCP scaffold (69.7% ± 6.6%).

Histomorphometric Analysis

Table 1 shows the total amount of new tissue (bone and fibrous tissue) new bone tissue, and new trabecular bone under the barriers. The data from both barriers were averaged because the effect of the scaffold and the addition of cells were similar.

The average percentage of new trabecular bone for the titanium barriers was 7.8% for the TCP with cells, 6.9% for the HA/TCP with cells, 8.1% for the TCP without cells, 8.8% for the HA/TCP without cells, and 7.0% for the group with the blood clot only.

There was no significant difference when the four groups were compared using the Kruskal-Wallis test for the new tissues found under the cup. The Mann-Whitney U test was performed to verify if there was an effect of the HA/TCP scaffold or an effect of adding cells. No significant difference was found for the new tissues when a HA/TCP scaffold was added or when cells were used.

Mann-Whitney U tests performed to verify if there was a significant difference between the four groups with a scaffold compared to the blood clot only did not reveal significant differences. There was a significant difference between the groups with a scaffold and the group with a blood clot only, for the amount of new bone tissue underneath the barriers (P <0.04).

DISCUSSION

An occlusive titanium barrier was used to establish the space necessary for bone augmentation by applying a scaffold material, cell-seeded or not, and a control group consisting of a blood clot only. During the last two decades, the proof of principle has been established that applying a space-providing membrane with an underlying blood clot is conducive for the formation of bone tissue, even beyond the skeletal envelope. The guided bone augmentation procedures have used the rabbit skull as a preclinical model to assess the formation of new bone when using an occlusive titanium barrier.[19] It has been established that a fully secluded space provided by a rigid subperiosteal titanium barrier filled with a blood clot is conductive for new bone ingrowth.[9,20] The latter study confirmed

that bone height augmentations up to 3 mm beyond the body envelope could be generated under an occlusive dome-shaped barrier placed on top of the calvarium of rabbits. In this study, we adopted this model to evaluate the contribution of two different scaffolds and autologous periosteum-derived cells to further enhance and speed up the bone augmentation process underneath a titanium barrier. However, no significant differences could be detected when cells were added to the carriers in this experiment. A small difference appeared under the anterior barriers between the scaffold groups and the controls only for newly formed bone.

The less successful results in the present experiment raise questions with regard to the use of periosteum-derived cells, their characterization, the density of the seeded cells needed to trigger bone formation, and the influence of the scaffold material. Our findings indicate rather disappointing results with periosteum-derived cells prepared under standard conditions. This may be related to a large number of variables, including the animal model, the characterization of the cells, or other experimental factors. The interaction of cells with their environment seems species specific. In an experiment by Solchaga et al.,[21] a high variability within each group of rabbits was also noticed. Their conclusions were that any experimental design using rabbit progenitor cells must include a large sample size to obtain statistically significant differences. Alternatively, species specificity can play a major role in the interpretation of our results. In a recent article by Eyckmans and Luyten,[22] ectopic bone formation by periosteum-derived cells retrieved from humans and rabbits was evaluated. They found that the human periosteum-derived cells displayed distinct growth characteristics and osteogenic differentiation capacity in vitro and in vivo compared to the rabbit periosteum-derived cells. In vivo, human and rabbit periosteal cells were seeded onto an HA/TCP scaffold and implanted subcutaneously on the back in the cervical region of nude mice and collected after 1.4 and 8 weeks of implantation. After 8 weeks of implantation, the scaffolds seeded with human periosteum-derived cells showed abundant *de novo* bone formation, whereas the scaffolds seeded with rabbit periosteal cells did not show new bone. Thus, rabbit periosteal-derived cells were not able to induce ectopic bone formation under these conditions. Their main conclusion indicated the potential limitations of the use of the rabbit as a preclinical model for cell-based treatments for bone repair.

It may be important to characterize the cells used in tissue-engineering procedures. In our study, cells were isolated from the periosteum, which is a bilayered membrane attached to the cortical bone and consisting of an outer fibrous layer and inner cambium layer. The cambium layer contains chondrocyte precursors[23] and osteoblast-like cells[24] that are highly active during callus formation.[25] Most likely, the cells derived from the cambium layer are the cells of interest for bone-engineering applications.[26] The cambium layer could have been damaged or separated from the entire periosteum remaining on the bone surface, thus stripping the fibrous layer only.[27] This may select a fibroblastic cell type and not the osteoprogenitors. However, in our study, the cambium layer and the fibrous layer were easily detected by histology, indicating that the harvesting procedure was acceptable (data not shown). In addition, the viability of periosteal explants is dependent on the time of harvesting and the time of transport. When harvested, they should be placed in a transport medium not more than 10 to 15 minutes after explantation. These conditions were fulfilled in our study.

The second step is the cell preparation and expansion. The periosteum is a heterogeneous tissue; when an enzymatic digestion of the periosteum is performed, the cells released from the tissue are a mixed population of cells from the fibrous and the cambium layer. Recently, Youn et al.[28] cultured the fibrous and the cambium layer separately, and a comparison was made between the two cell cultures. Each layer of the periosteum had specific phenotypic characteristics in a monolayer culture. They demonstrated that the rabbit periosteal fibroblasts proliferated faster than the cambium-derived cells in vitro, whereas the osteogenic potential of the cambium-derived cells was much higher compared to the fibroblasts. These data suggest that the culture conditions in our study may not have preserved the cambium-derived population, resulting in a cell population that predominantly contained periosteal fibroblasts with reduced bone augmentation process in vivo. However, there are indications that even those heterogeneous cell populations can induce bone formation. In previous studies[10,11] with these heterogeneous cells, the periosteum-derived cells had the potential to differentiate into osteoblasts in vivo. Thus, we believe that the cells used in our experiment should have been sufficiently osteogenic to achieve the intended bone augmentation because it has been shown that these periosteum-derived cell populations grown under similar conditions are capable of healing critical-sized defects in the skull[29] or the tibia of rabbits.[13]

The engraftment of viable cells in the scaffold and the differentiation potential into the osteogenic cell lineage with bone formation also are unpredictable factors. The choice of the scaffolds, namely the use of calcium phosphate scaffolds, may also have jeopardized the bone formation in our experiment. According to LeGeros,[16] the presence of a mineralized scaffold microenvironment is required for the onset

of bone formation by committed human cells by creating and maintaining a space that controls cell migration, proliferation, and differentiation as well as vascular ingrowth in the scaffold. The material to be used for periosteal cells should meet the following criteria: usage in bone replacement procedures in humans, biocompatibility, a known resorption rate, and a high affinity for cells. For these reasons we chose a calcium phosphate carrier, which is made of HA, TCP, and collagen, and a β-TCP carrier.

Both materials used are commercially available and are already used as jaw graft material for spinal fusions and other orthopedic indications.[30,31] TCP§§§§§§ was approved by the U.S. Food and Drug Administration in late 2000[32] for use in traumatic or surgically created defects in the extremities, pelvis, and spine. HA/TCP‖‖‖‖‖‖‖‖ was approved in 1993[33] and can be used as a paste or in a strip. Second, these bone substitutes are similar to the composition of bone mineral and favor the development of a direct, adherent, and strong bond to bone. Third, these calcium phosphate materials are osteoconductive,[6] supporting tissue ingrowth, the growth of osteoprogenitor cells, and the development of new bone. It was shown that calcium phosphates allow attachment and migration of bone cells, leading to new bone in direct apposition with the calcium phosphate particles. Fourth, the cell-mediated biodegradation that occurs is greater for TCP than for the HA material. The resorption characteristics of tricalcium phosphates are intended to match the course of natural bone healing, and it resorbs in parallel with bone ingrowth. New bone formation in the TCP material was characterized by a resorption of the calcium phosphate (CaP) structure together with the deposition of new bone initiating from the original skull bone. However, in the HA/TCP scaffold, new bone grew in between the ceramic particles, in close contact with them, and throughout the entire scaffold surface, and it was not always associated with a resorption as seen in the TCP. Finally, CaP compounds have a high affinity for mesenchymal stem cells. Periosteum-derived cells were first studied in vivo to evaluate the bone augmentation potential in nude mice whereby the cells were seeded onto ceramic scaffolds.[34]

However, the addition of the rabbit periosteum-derived cells seeded onto both scaffolds did not result in the enhancement of bone formation underneath the titanium barriers. Bone formation occurring under the barriers in the two different CaP-based scaffolds in this rabbit skull site could be due to the recruitment of mesenchymal stem cells originating from the bone marrow in between the two tabulae of the skull and the osteoconductive properties of the two scaffolds. However, further cell tracking studies are needed to confirm this hypothesis.

A last striking phenomenon was the presence of seromas in 73% of the cups not related to the treatment modality. A seroma is defined as a sterile accumulation of serum in a circumscribed dead space in the tissue without detectable cells or fibers in the center. These seromas of bone voids were observed in different experimental protocols, such as in canine periodontal and alveolar defects or in peri-implant bone regeneration.[35,36] In these experiments, the bone voids appeared as empty vacuoles without a definable matrix but with visible new bone formation at the periphery of the seromas and the possibility of a gradual refilling with bone.[36]

CONCLUSIONS

Our findings emphasize that understanding the mechanism of action will be critical if we want to bring tissue-engineering approaches into successful and clinically relevant treatments. A very accurate balance is probably required between the proper cell populations, the adequate environment, and the matching scaffold. Despite the fact that the proof of principle of bone tissue engineering using periosteum-derived cells and HA carriers has been demonstrated,[15] a better understanding of the interactions of cells with the microenvironment provided by the carrier structure and the implantation site is required to make a tissue-engineering product consistent and reproducible. Moreover, the choice of the appropriate preclinical model for the development of tissue-engineering protocols for specific applications envisioned in humans remains a major challenge. In this perspective, the present choice of rabbit periosteal cells can be challenged. The interaction of cells with their microenvironment seems to be species specific and can explain our poor results when adding cells.

ACKNOWLEDGMENTS

Dr. Maréchal is a project leader and researcher scientist at TiGenix, Heverlee, Belgium. The authors thank P. Avontroodt for his contribution to the rabbit surgery. This work was supported by the Catholic University Leuven grant IDO/00/006. The authors report no conflicts of interest related to this study.

REFERENCES

1. Breine U, Brånemark P-I. Reconstruction of alveolar jaw bone: An experimental and clinical study of immediate and preformed autologous bone grafts in combination with osseointegrated implants. *Scand J Plast Reconstr Surg* 1980;14:23-48.

§§§§§§ Vitoss, Orthovita.
‖‖‖‖‖‖‖‖ Collagraft, Neucoll.

2. van Steenberghe D, Quirynen M, Svensson B, Brånemark P-I. Clinical examples of what can be achieved in anatomically severely compromised patients. *Periodontol 2000* 2003;33:90-104.
3. Becker W, Becker BE, Caffesse R. A comparison of demineralized freeze-dried bone and autologous bone to induce bone formation in human extraction sockets. *J Periodontol* 1994;65:1128-1133.
4. Mellonig JT, Prewett AB, Moyer MP. HIV inactivation in a bone allograft. *J Periodontol* 1992;63:979-983.
5. Nasr HF, Aichelmann-Reidy ME, Yukna RA. Bone and bone substitutes. *Periodontol 2000* 1999;19:74-86.
6. Nagahara K, Isogai M, Shibata K, Meenaghan MA. Osteogenesis of hydroxyapatite and tricalcium phosphate used as a bone substitute. *Int J Oral Maxillofac Implants* 1992;7:72-79.
7. Klein CP, Driessen AA, de Groot K, van den Hooff A. Biodegradation behavior of various calcium phosphate materials in bone tissue. *J Biomed Mater Res* 1983;17:769-784.
8. Simion M, Trisi P, Piatelli A. Guided bone regeneration for vertical ridge augmentation associated with osseointegrated implants. In: Nevins M, Mellonig J, eds. *Implant Therapy*. Chicago: Quintessence; 1998:99-110.
9. van Steenberghe D, Johansson C, Quirynen M, Molly L, Albrektsson T, Naert I. Bone augmentation by means of a stiff occlusive titanium membrane. A study in rabbits and humans. *Clin Oral Implants Res* 2003; 14:63-71.
10. Nakahara H, Bruder SP, Goldberg VM, Caplan AI. In vivo osteochondrogenic potential of cultured cells derived from the periosteum. *Clin Orthop Relat Res* 1990;259:223-232.
11. Nakahara H, Bruder SP, Haynesworth SE, et al. Bone and cartilage formation in diffusion chambers by subcultured cells derived from the periosteum. *Bone* 1990;11:181-188.
12. Puelacher WC, Vacanti JP, Ferraro NF, Schloo B, Vacanti CA. Femoral shaft reconstruction using tissue-engineering growth of bone. *Int J Oral Maxillofac Surg* 1996;25:223-228.
13. Takushima A, Kitano Y, Kiyonori H. Osteogenic potential of cultured periosteal cells in a distracted bone gap in rabbits. *J Surg Res* 1998;78:68-77.
14. De Bari C, Dell'Accio F, Vanlauwe J, et al. Mesenchymal multipotency of adult human periosteal cells demonstrated by single-cell lineage analysis. *Arthritis Rheum* 2006;54:1209-1221.
15. Vacanti CA, Bonassar LJ, Vacanti MP, Shufflebarger J. Replacement of an avulsed phalanx with tissue-engineered bone. *N Engl J Med* 2001;344:1511-1514.
16. LeGeros RZ. Properties of osteoconductive biomaterials: Calcium phosphates. *Clin Orthop Relat Res* 2002;395:81-98.
17. Nishimura I, Shimizu Y, Ooya K. Effects of cortical bone perforation on experimental guided bone regeneration. *Clin Oral Implants Res* 2004;15:293-300.
18. Data Sciences International. Seromas: Cause and management. Available at: http://www.datasci.com/information/technotes/392-0027-002.asp.
19. Lundgren D, Lundgren AK, Sennerby L, Nyman S. Augmentation of intramembranous bone beyond the skeletal envelope using an occlusive titanium barrier. An experimental study in the rabbit. *Clin Oral Implants Res* 1995;6:67-72.
20. Schmid J, Hämmerle CHF, Olah AJ, Lang NP. Membrane permeability is unnecessary for guided tissue generation of new bone. An experimental study in the rabbit. *Clin Oral Implants Res* 1994;5:125-130.
21. Solchaga LA, Johnstone B, Yoo JU, Goldberg VM, Caplan AI. High variability in rabbit bone marrow-derived mesenchymal cell preparations. *Cell Transplant* 1999;8:511-519.
22. Eyckmans J, Luyten FP. Species specificity of ectopic bone formation by periosteum-derived mesenchymal progenitor cells. *Tissue Eng* 2006;12:2203-2213.
23. Ito Y, Fitzsimmons JS, Sanyal A, Mello MA, Mukherjee N, O'Driscoll SW. Localization of chondrocyte precursors in periosteum. *Osteoarthritis Cartilage* 2001;9: 215-223.
24. Stafford HJ, Roberts MT, Oni OO, Hay J, Gregg P. Localization of bone forming cells during fracture healing by osteocalcin immunocytochemistry: An experimental study of the rabbit tibia. *J Orthop Res* 1994;12:29-39.
25. Kojimoto H, Yasui N, Goto T, Matsuda S, Shimomura Y. Bone lengthening in rabbits by callus distraction. The role of periosteum and endosteum. *J Bone Joint Surg Br* 1988;70:543-549.
26. Hutmacher DW, Sittinger M. Periosteal cells in bone tissue engineering. *Tissue Eng* 2003;9(Suppl. 1):S113-S126.
27. O'Driscoll SW, Fitzsimmons JS. The importance of procedure specific training in harvesting periosteum for chondrogenesis. *Clin Orthop Relat Res* 2000;380: 269-278.
28. Youn I, Suh J-KF, Nauman EA, Jones DG. Differential phenotypic characteristics of heterogeneous cell population in the rabbit periosteum. *Acta Orthop* 2005;76: 442-450.
29. Breitbart AS, Grande DA, Kessler R, Ryaby JT, Fitzsimmons RJ, Grant RT. Tissue engineering bone repair of calvarial defects using cultured periosteal cells. *Plast Reconstr Surg* 1998;101:567-574.
30. Linovitz RJ, Peppers TA. Use of an advanced formulation of beta-tricalcium phosphate as a bone extender in interbody lumbar fusion. *Orthopedics* 2002;25: S585-S589.
31. Cornell CN, Lane JM, Chapman M, et al. Multicenter trial of Collagraft as bone graft substitute. *J Orthop Trauma* 1991;5:1-8.
32. FDA. Center for Devices and Radiological Health. Available at: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfPMA/PMA.cfm?ID=4978.
33. FDA. Center for Devices and Radiological Health. Available at: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfPMN/pmn.cfm?ID=119369.
34. Nakahara H, Goldberg VM, Caplan AI. Culture-expanded human periosteal-derived cells exhibit osteochondral potential in vivo. *J Orthop Res* 1991;9:465-476.
35. Wikesjö UM, Qahash M, Thomson RC, et al. rhBMP-2 significantly enhances guided bone regeneration. *Clin Oral Implants Res* 2004;15:194-205.
36. Jovanovic SA, Hunt DR, Bernard GW, et al. Long-term functional loading of dental implants in rhBMP-2 induced bone. A histologic study in the canine ridge augmentation model. *Clin Oral Implants Res* 2003;14: 793-803.

Correspondence: Dr. Marina Maréchal, TiGenix, Romeinsestraat 12 Bus 2, 3001 Heverlee, Belgium. Fax: 32-0-16-396070; e-mail: marina.marechal@tigenix.com.

Submitted January 22, 2007; accepted for publication November 14, 2007.