Patrick H. Warnke
Timothy Douglas
Sureshan Sivananthan
Jörg Wiltfang
Ingo Springer
Stephan T. Becker

Tissue engineering of periosteal cell membranes *in vitro*

Authors' affiliations:
Patrick H. Warnke, Timothy Douglas, Jörg Wiltfang, Ingo Springer, Stephan T. Becker, Department of Oral and Maxillofacial Surgery, University of Kiel, Kiel, Germany
Sureshan Sivananthan, Epsom and St. Helier University Hospitals NHS Trust and South West London Elective Orthopaedic Centre, University of London, London, UK
Patrick H. Warnke, Faculty of Health Sciences and Medicine, Bond University, Gold Coast, Qld, Australia

Correspondence to:
Patrick H. Warnke
Department of Oral and Maxillofacial Surgery
University of Kiel
Haus 26, Arnold-Heller-Strasse 3
24105 Kiel
Germany
Tel.: +49 431 597 2821
Fax: +49 431 597 4084
e-mail: warnke@mkg.uni-kiel.de

Date:
Accepted 11 January 2009

To cite this article:
Warnke PH, Douglas T, Sivananthan S, Wiltfang J, Springer I, Becker ST. Tissue engineering of periosteal cell membranes *in vitro*.
Clin. Oral Impl. Res. **20**, 2009; 761–766.
doi: 10.1111/j.1600-0501.2009.01709.x

Key words: collagen membrane, periosteum, stem cell, tissue engineering

Abstract:

Objectives: The cultivation of bone is a major focus in tissue engineering and oral implantology. Without a periosteal layer, instant or rapid development of a substantial cortical layer is unlikely for engineered bone grafts. The aim of this study was to test the ability of four collagen membranes to support and promote the proliferation of human periosteal cells.

Materials and methods: Human periosteum cells were cultured using an osteogenic medium consisting of Dulbecco's modified Eagle's medium supplemented with fetal calf serum, penicillin, streptomycin and ascorbic acid at 37°C with 5% CO_2. Four collagen membranes served as scaffolds: Bio-Gide, Chondro-Gide, Tutodent and Ossix Plus. Cell vitality was assessed by fluorescin diacetate (FDA) and propidium iodide (PI) staining, biocompatibility with LDH and BrdU, MTT, WST tests and scanning electron microscopy (SEM).

Results: After 24 h, all probes showed viable periosteal cells. All biocompatibility tests revealed that proliferation on all membranes after treatment with eluate from membranes after a 24-h immersion in a serum-free cell culture medium was similar to the controls. Periosteal cells formed layers covering the surfaces of all four membranes 7 days after seeding in SEM.

Conclusion: It can be concluded from our data that the collagen membranes can be used as scaffolds for the cultivation of periosteum layers with a view to creating cortical bone using tissue-engineering methods.

The cultivation of large bone replacements is a major focus in tissue engineering. *In vivo* tissue-engineering techniques such as intramuscular endocultivation, where the patient serves as his own bioreactor, have yielded customized and vascularized bone grafts that have been used to reconstruct the patient's skeleton (Warnke et al. 2004, 2006). Other *in vitro* studies have produced artificial matrices with bioactive cytokines or preseeded osteoblasts as bone graft materials with high potential (Mai et al. 2006; Claase et al. 2007).

All these engineered bone grafts have no periosteum at the beginning. Without a periosteum the instant or rapid development of a substantial cortical layer is unlikely (Li et al. 2004). The promotion of cortical bone growth may also increase the stability of dental implants in clinical practice.

However, cultivation of periosteal margins for *in vitro* cultivated bone from a tissue culture has rarely been reported, although the osteoinductive and nutritious capabilities of the periosteum are well

© 2009 John Wiley & Sons A/S

known (Hutmacher 2000). Periosteal cells proliferate faster than marrow stromal cells (Agata et al. 2007). Transplants of periosteal cells treated with BMP-2 after pretreatment with bFGF formed more new bone than did marrow stromal cells (Agata et al. 2007). Periosteal cells can be cultivated (Breitbart et al. 1998) and proliferate on resorbable membranes (Arnold et al. 2002). It is remarkable that the periosteum contains many osteoprogenitor cells that showed high bone formation rates in animal models (Vogelin et al. 2005). It is also a source for mesenchymal stem cells. Thus, periosteal cells may have important advantages for bone tissue engineering (Zhang et al. 2008) and oral implantology, as they are able to induce bone formation and may induce a cortical margin and reduce bone overgrowth outside the scaffolds. The natural periosteum has a bilayered structure. The stratum fibrosum provides mechanical stability for inserting ligaments, whereas even in adults the stratum osteogenium hosts fibroblasts, mesenchymal stem cells and already osteoprogenitor cells that are capable of differentiation into osteoblasts. The mesenchymal stem cells may offer another source for recruitment of endothelial cells for fast development of a vascular system inside engineered bone.

Periosteum can be harvested easily during oral surgery. Periosteal cell cultivation and proliferation to enhance cell numbers can be performed *in vitro* (Breitbart et al. 1998).

As a scaffold for periosteal cell cultivation and transplantation, collagen membranes may be suitable. They are popular materials for guided bone regeneration (GBR) in oral surgery (Rothamel et al. 2004). Because of their biodegradability, a second surgical procedure for their removal is unnecessary. Also, they are less susceptible to bacterial colonization than non-degradable barrier membrane materials such as gore-tex (Rothamel et al. 2005).

The aim of this study was to test the four commercially available collagen membranes Bio-Gide, Chondro-Gide (both Geistlich Pharma AG, Wolhusen, Switzerland), Tutodent (Tutogen Medical GmbH, Neunkirchen am Brand, Germany) and Ossix Plus (3i, Karlsruhe, Germany) for their biocompatibility and ability to support and promote the proliferation of human periosteal cells.

Material and methods

Isolation and cultivation of cells from periosteum

Human periosteum biopsies were harvested from patients during the course of oral surgery (wisdom tooth removal). The study was approved by the ethics board of the University of Kiel, D 417/07). The cells were cultivated using an osteogenic medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100IE penicillin/ml, 100 µg streptomycin/ml and 1 mmol/l ascorbic acid at 37°C with 5% CO_2. Cell seeding was performed after the second passage. During passaging, cells were detached from 75 cm^2 cell culture flasks using 5 ml of a 0.05% trypsin solution in phosphate-buffered saline (PBS). After a 1 : 1 dilution of the cell suspension with DMEM containing 10% FCS and centrifugation at 3200*g* for 3 min, cells were resuspended in DMEM containing 10% FCS, counted and reseeded at a density of 10^5 cells per 75 cm^2 cell culture flask. Cells were cultured in the same medium used for cell seeding in a humidified atmosphere with 5% CO_2 at 37°C. Medium change took place every 3 days.

Membranes

The following four collagen membranes served as scaffolds for cultivation of the cells: Bio-Gide (porcine collagen types I and III, non-crosslinked), Chondro-Gide (porcine collagen types I and III, bilayer; both Geistlich Pharma AG), Tutodent (non-crosslinked bovine type I collagen, bilayered; Tutogen Medical GmbH) and Ossix Plus (bovine collagen type 1, enzymatic-crosslinked; 3i). The membranes were divided into quadratic pieces of side length 7 mm and placed in 24-well cell culture plates (Nunc GmbH, Langensel bold, Germany). Cells were seeded on membrane pieces at a density of 10^4 cells/well. Cells were cultured on membrane pieces in 2000 µl of the same medium used for cell seeding in a humidified atmosphere with 5% CO_2 at 37°C. Medium change took place every 3 days. At these points, cultures were checked microscopically.

Assessment of cell vitality

Cell vitality was assessed by Fluorescin Diacetate (FDA) and Propidium Iodide (PI) staining. Staining was performed on cells cultured in an eluate from membranes after a 24-h incubation in cell culture medium. 5×10^3 cells in cell culture medium with 10% FCS were seeded on eight-well objectives. After 1 day of culture, 200 µl eluate from membranes immersed in serum-free cell culture medium for 24 h was added to cells. After a 24-h incubation at 37°C and 5% CO_2, cells were rinsed with PBS and immersed in an FDA solution prepared by diluting 30 µl × 1 mg FDA/ml acetone in 10 ml PBS. After incubation for 15 min at 37°C in the dark, the FDA solution was removed by suction and replaced with a PI solution prepared by diluting 500 µl × 1 mg/ml PI in 10 ml PBS. After incubation for 2 min at room temperature in the dark, scaffolds were rinsed twice in PBS. While still immersed in PBS, scaffolds were then subjected to fluorescence microscopy with excitation at 488 nm and detection at 530 nm (FDA, green) and 620 nm (PI, red).

Biocompatibility tests

LDH and BrdU, MTT and WST tests

LDH tests can show cell death and lysis. Cells were seeded in 96-well cell culture plates (Nunc) in 100 µl DMEM at a concentration of 5×10^3 cells/well. After a 24-h culture in a humidified atmosphere with 5% CO_2 at 37°C, medium was removed and replaced with 150 µl eluate from the membranes. Cells cultured in 2% Triton-X-100 in serum-free DMEM served as high controls. Cells cultured in serum-free DMEM served as low controls. After a 24-h incubation, 100 µl eluate was transferred to another 96-well cell culture plate. Extracellular LDH activity was measured with the help of an LDH detection kit (Roche Diagnostics, Mannheim, Germany, Catalogue no. 11644793001). Absorbance was measured at 490 nm. The remaining 50 µl eluate per well remaining in the cell culture plate was removed and replaced with 100 µl DMEM containing 10% FCS, penicillin/ml, 100 µg streptomycin/ml and 1 mmol/l ascorbic acid. After 7 days of incubation, proliferation was measured with the help of a BrdU Cell Proliferation ELISA kit (Roche Diagnostics, Catalogue no. 11647229001). Absorbance was measured at 450 nm.

© 2009 John Wiley & Sons A/S

After a 24-h incubation with an eluate from the membranes, proliferation was assessed with the aid of an MTT Cell Proliferation Kit (Roche Diagnostics, Catalogue no. 11465007001). Calibration curves of 0.16–10 × 10^3 cells/well served as standards. Absorbance was measured at 450 nm.

As mentioned previously, membrane pieces were seeded at a density of 10^4 cells/piece. After 7 days of culture in 2000 µl cell culture medium, proliferation was assessed with the aid of a Cell Proliferation Reagent WST-1 (Roche Diagnostics, Catalogue no. 11644807001). Briefly, 200 µl WST-1 reagent was added to each well at a 1 : 10 ratio to cell culture medium. After a 4-h incubation in a humidified atmosphere with 5 % CO_2 at 37°C, medium was transferred to 96-well plates and absorbance was measured at 450 nm. Cells cultured in wells without membrane pieces at a density of 10^4 cells/well served as controls.

Scanning Electron Microscopy (SEM) examinations

SEM investigations were carried out 1 week after cell seeding using an XL30CP device (Phillips Electron Optics GmbH, Kassel, Germany) operating at 10–25 kV, as used by Yang et al. (2006). As preparation for the SEM investigation, cell-seeded membranes and membranes without cells as control were first rinsed using PBS to remove cell culture medium. Cells were then fixed using 3 % glutaraldehyde in PBS at pH 7.4 for 24 h. After removal of the glutaraldehyde solution, cells were dehydrated by incubating scaffolds in a series of ethanol solutions of increasing concentration. Scaffolds were immersed for 5 min in each of the following ethanol solutions: 50%, 60%, 70%, 80%, 90% and 100%. Subsequently, critical point drying was performed using a K850 critical point dryer (Emitech, EM Technologies Ltd., Ashford, UK), followed by gold sputtering with an SCD 500 device (CAL-Tec, Ashford, UK).

Statistical evaluation

Means of the eight trials per assay and standard deviations were calculated and are presented in figures.

Results

Assessment of cell vitality on membranes

After 24 h, all probes showed viable periosteal cells. The pronounced green color of the cells due to FDA staining demonstrates their vitality on all membranes, while the absence of a red color despite PI staining indicated that no cells died as a result of eluate from any membrane. These results are illustrated in Fig. 1.

Biocompatibility tests

In the case of the LDH test, cytotoxicity on all membranes after treatment with an eluate from membranes after a 24-h immersion in a serum-free cell culture medium was similar to the low control (zero cytotoxicity) and significantly different from the high control (maximum cytotoxicity). The results are presented in Fig. 2.

Fig. 3 shows MTT test results, BrdU test results are presented in Fig. 4 and WST test results are shown in Fig. 5. The MTT test gives a measure of cellular metabolic activity dependent on living cells, proliferation, viability and cytotoxicity. At a later point than MTT, the WST test indicates the metabolic activity of cells. BrdU tests show cell proliferation by incorporation of BrdU during DNA synthesis. In the case of all three tests, proliferation on all membranes after treatment with eluate from membranes after a 24-h immersion in a serum-free cell culture medium was similar to controls. These results demonstrate the good biocompatibility of all membranes for human periosteal cells.

SEM investigations of cell morphology on membranes

Periosteal cells formed layers covering the surfaces of all four membranes 7 days after seeding. The close-up images in Fig. 6 show that all membranes were almost completely covered by cells, which had an elongated morphology with numerous cell pseudopodia, suggesting good biocompatibility. Representative SEM images of human periosteal cells seeded on membranes can be seen in Fig. 6.

Discussion

The visionary goal of this research is to grow periosteal membranes for tissue engineering and GBR. This first study demonstrates the biocompatibility of the collagen-based membranes Bio-Gide, Chondro-Gide (both Geistlich Pharma AG), Tutodent (Tutogen Medical GmbH)

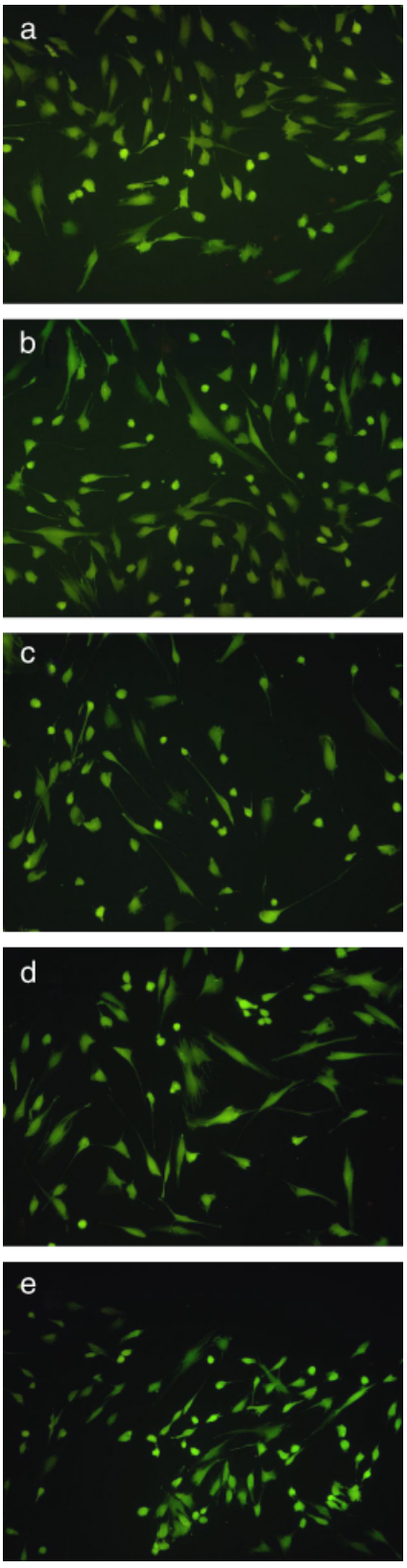

Fig. 1. Fluorescence microscopy images of human periosteal cells after staining with fluorescin diacetate (FDA) and propidium iodide (PI): note the green color due to staining with FDA, indicating living cells. The lack of a red color despite staining with PI indicates absence of dead cells. (a) Bio-Gide; (b) Chondro-Gide; (c) Tutodent; (d) Ossix Plus; and (e) Control.

© 2009 John Wiley & Sons A/S

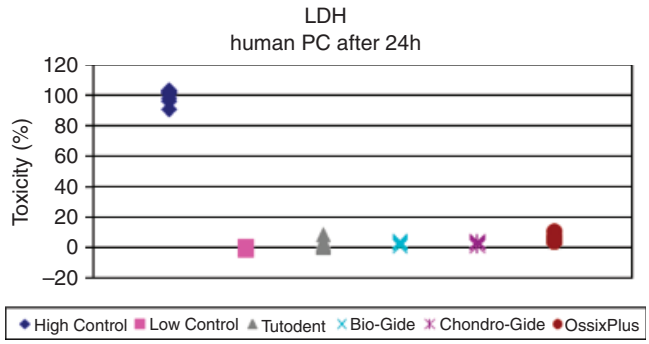

Fig. 2. LDH test of cells from a human periosteum on Tutodent, Bio-Gide, Chondro-Gide and Ossix Plus. Toxicity is minimal and around the low control for all membranes.

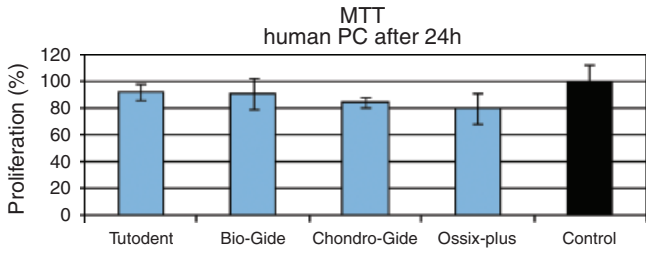

Fig. 3. MTT test of cells from a human periosteum on Bio-Gide, Chondro-Gide, Tutodent and Ossix Plus showing high proliferation rates for all the membranes tested. Polystyrene served as a control.

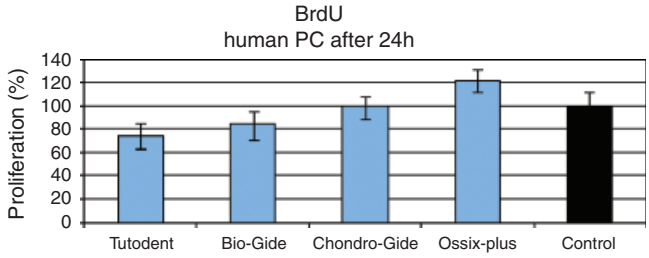

Fig. 4. Proliferation (BrdU) test of cells from a human periosteum on Bio-Gide, Chondro-Gide, Tutodent and Ossix Plus. Note the high cell proliferation values for all membranes. Polystyrene served as a control.

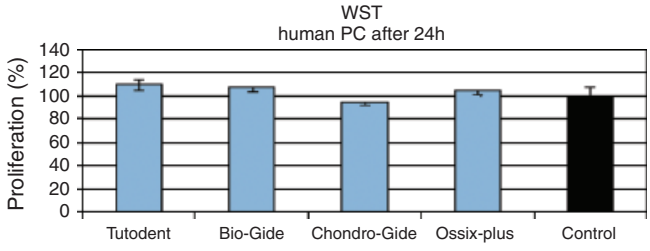

Fig. 5. Successful WST test results of cells from a human periosteum on Bio-Gide, Chondro-Gide, Tutodent and Ossix Plus. Polystyrene served as a control.

and Ossix Plus (31) and their ability to support and promote the proliferation of human periosteal cells.

On the basis of the results of the SEM investigations, the cell vitality staining and the biocompatibility tests (LDH, MTT, BrdU, and WST), it can be concluded that the Bio-Gide, Chondro-Gide, Tutodent and Ossix Plus membranes are biocompatible for cells derived from human periosteum. These cells are important for GBR on the one hand. Collagen membranes are popular materials for GBR in oral surgery (Rothamel et al. 2004). The results are in accordance with other studies, where different cell types were investigated. Rothamel et al. (2004) evaluated the biocompatibility of collagen membranes in cultures of human periodontal ligament (PDL) fibroblasts and human osteoblast-like cells. He concluded that Bio-Gide, Tutodent and Ossix Plus promoted the attachment and proliferation of human PDL fibroblasts and human SaOs-2 osteoblasts and that collagen is chemotactic for PDL fibroblasts (Rothamel et al. 2004). In cultures of human osteoblast-like cells, cells were able to proliferate on collagen membranes (Bilir et al. 2007). Cell attachment of rat PDL-derived cells is different on different collagen devices. Membrane materials influence cell proliferation and differentiation in the process of periodontal tissue regeneration (Takata et al. 2001a). The collagen fibers of Bio-Gide are believed to support osteogenic differentiation *in vivo* (Taguchi et al. 2005). Using mouse osteoprogenitor cells, collagen is a favorable scaffold for osteoblastic cells to proliferate (Takata et al. 2001b). Also, they are less susceptible to bacterial colonization than non-degradable barrier membrane materials such as gore-tex (Rothamel et al. 2005), although the adherence of bacterial strains to collagen membranes is high (Sela et al. 1999).

There is growing interest in the use of multipotent mesenchymal stem cells and so-called osteoprogenitor cells harvested from the bone marrow or the periosteum for *in vitro* and *in vivo* osteogenesis. These cells can be differentiated to several cell types by the application of different growth and differentiation factors and suitable matrices as cell adhesives (Kubler & Urist 1991; Iwasaki et al. 1994; Lecanda et al. 1997; Chang et al. 2004; Warnke et al. 2004). The most important growth factors for differentiation of mesenchymal stem cells to osteogenic cells are BMP-2 and BMP-7 out of the TGF-β superfamily. For clinical research, the application of these cytokines for regeneration of bony defects is important.

One way to replace bony structures is the direct application of BMP combined with different matrices in a defect. In animal experiments, bone regeneration was significantly increased (Wiltfang et al. 1996). Another method, even for larger defects, is heterotopic bone regeneration by means of endocultivation (Terheyden et al. 2001a,

© 2009 John Wiley & Sons A/S

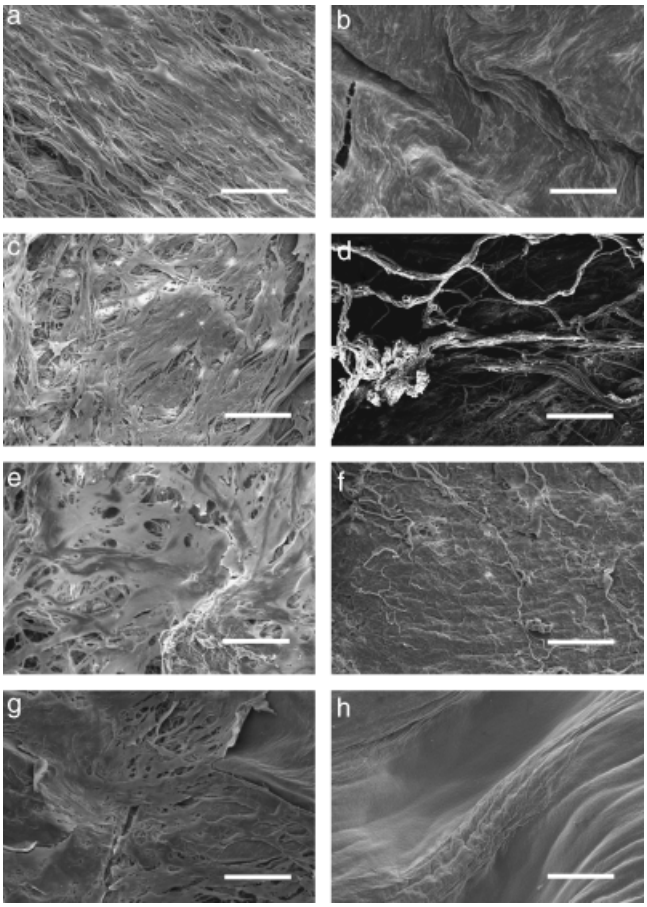

Fig. 6. Close-up SEM images of membranes seeded with human periosteal cells and controls 7 days after seeding. The membranes were almost completely covered by cells, with an elongated morphology and numerous cell pseudopodia, suggesting good biocompatibility (a) Bio-Gide with cells; (b) Bio-Gide control without cells; (c) Chondro-Gide with cells; (d) Chondro-Gide control without cells; (e) Tutodent with cells; (f) Tutodent control without cells; (g) Ossix Plus with cells; and (h) Ossix Plus control without cells (scale bar = 20 μm).

2001b; Warnke et al. 2004). One phenomenon of endocultivation is the missing cortical layer in the early stages and exceeding bone development in some areas. This complicates the production of perfect-fit replacements. A perfect fit is essential to enhance fast integration and mechanical stability of the cultivated bone inside the recipient area. Our aim is to endocultivate customized vascularized bone transplant with a periosteal layer to avoid these minor disadvantages. Collagen membranes may serve as a scaffold to transplant cultured periosteal cells that form a periosteum on the transplant.

Future work could involve the incorporation of other extracellular matrix components such as chondroitin sulfate, which has been reported to have a beneficial effect on cell proliferation as a component of a collagen-based membrane (Marinucci et al. 2003). Another option is the use of bilayered membranes modified for the generation of stratum fibrosum and osteogenicum or the application of cytokines for differentiation of osteoblasts. Cell-seeded collagen membranes may also stabilize chondrocytes or stem cells after articular chondrocyte injection in orthopedic surgery for reconstruction of cartilage defects in joints (Funayama et al. 2008).

Conclusion

It can be concluded from our data that the membranes Bio-Gide, Chondro-Gide, Tutodent and Ossix Plus are biocompatible for cells derived from a human periosteum. On the one hand, this is an important issue for GBR. On the other, the positive results pave the way for the use of the membranes as scaffolds for the generation of periosteum layers to create cortical bone using tissue-engineering methods.

Acknowledgements: The authors thank the European Union for financial support within the framework of the MyJoint Project (FP-6 NEST 028861) and G. Otto for excellent technical assistance. We thank Professor Eugene Sherry, Faculty of Health Sciences and Medicine, Bond University, Australia, for inspiring discussions while designing and performing this study.

References

Agata, H., Asahina, I., Yamazaki, Y., Uchida, M., Shinohara, Y., Honda, M.J., Kagami, H. & Ueda, M. (2007) Effective bone engineering with periosteum-derived cells. *Journal of Dental Research* **86**: 79–83.

Arnold, U., Lindenhayn, K. & Perka, C. (2002) *In vitro*-cultivation of human periosteum derived cells in bioresorbable polymer-TCP-composites. *Biomaterials* **23**: 2303–2310.

Bilir, A., Aybar, B., Tanrikulu, S.H., Issever, H. & Tuna, S. (2007) Biocompatibility of different barrier membranes in cultures of human CRL 11372 osteoblast-like cells: an immunohistochemical study. *Clinical Oral Implants Research* **18**: 46–52.

Breitbart, A.S., Grande, D.A., Kessler, R., Ryaby, J.T., Fitzsimmons, R.J. & Grant, R.T. (1998) Tissue engineered bone repair of calvarial defects using cultured periosteal cells. *Plastic and Reconstructive Surgery* **101**: 567–574.

Chang, S.C., Chuang, H., Chen, Y.R., Yang, L.C., Chen, J.K., Mardini, S., Chung, H.Y., Lu, Y.L., Ma, W.C. & Lou, J. (2004) Cranial repair using BMP-2 gene engineered bone marrow stromal cells. *Journal of Surgical Research* **119**: 85–91.

Claase, M.B., de Bruijn, J.D., Grijpma, D.W. & Feijen, J. (2007) Ectopic bone formation in cell-seeded poly(ethylene oxide)/poly(butylene terephthalate) copolymer scaffolds of varying porosity. *Journal of Materials Science: Materials in Medicine* **18**: 1299–1307.

Funayama, A., Niki, Y., Matsumoto, H., Maeno, S., Yatabe, T., Morioka, H., Yanagimoto, S., Taguchi, T., Tanaka, J. & Toyama, Y. (2008) Repair of full-thickness articular cartilage defects using inject-

© 2009 John Wiley & Sons A/S

able type II collagen gel embedded with cultured chondrocytes in a rabbit model. *Journal of Orthopaedic Science* **13**: 225–232.

Hutmacher, D.W. (2000) Scaffolds in tissue engineering bone and cartilage. *Biomaterials* **21**: 2529–2543.

Iwasaki, M., Nakahara, H., Nakase, T., Kimura, T., Takaoka, K., Caplan, A.I. & Ono, K. (1994) Bone morphogenetic protein 2 stimulates osteogenesis but does not affect chondrogenesis in osteochondrogenic differentiation of periosteum-derived cells. *Journal of Bone and Mineral Research* **9**: 1195–1204.

Kubler, N. & Urist, M.R. (1991) Allogenic bone and cartilage morphogenesis. Rat BMP *in vivo* and *in vitro*. *Journal of Craniomaxillofacial Surgery* **19**: 283–288.

Lecanda, F., Avioli, L.V. & Cheng, S.L. (1997) Regulation of bone matrix protein expression and induction of differentiation of human osteoblasts and human bone marrow stromal cells by bone morphogenetic protein-2. *Journal of Cellular Biochemistry* **67**: 386–396.

Li, M., Amizuka, N., Oda, K., Tokunaga, K., Ito, T., Takeuchi, K., Takagi, R. & Maeda, T. (2004) Histochemical evidence of the initial chondrogenesis and osteogenesis in the periosteum of a rib fractured model: implications of osteocyte involvement in periosteal chondrogenesis. *Microscopy Research and Technique* **64**: 330–342.

Mai, R., Hagedorn, M.G., Gelinsky, M., Werner, C., Turhani, D., Spath, H., Gedrange, T. & Lauer, G. (2006) Ectopic bone formation in nude rats using human osteoblasts seeded poly(3)hydroxybutyrate embroidery and hydroxyapatite-collagen tapes constructs. *Journal of Craniomaxillofacial Surgery* **34** (Suppl. 2): 101–109.

Marinucci, L., Lilli, C., Guerra, M., Belcastro, S., Becchetti, E., Stabellini, G., Calvi, E.M. & Locci, P. (2003) Biocompatibility of collagen membranes crosslinked with glutaraldehyde or diphenylphosphoryl azide: an *in vitro* study. *Journal of Biomedical Materials Research Part A* **67**: 504–509.

Rothamel, D., Schwarz, F., Sager, M., Herten, M., Sculean, A. & Becker, J. (2005) Biodegradation of differently cross-linked collagen membranes: an experimental study in the rat. *Clinical Oral Implants Research* **16**: 369–378.

Rothamel, D., Schwarz, F., Sculean, A., Herten, M., Scherbaum, W. & Becker, J. (2004) Biocompatibility of various collagen membranes in cultures of human PDL fibroblasts and human osteoblast-like cells. *Clinical Oral Implants Research* **15**: 443–449.

Sela, M.N., Steinberg, D., Klinger, A., Krausz, A.A. & Kohavi, D. (1999) Adherence of periodontopathic bacteria to bioabsorbable and non-absorbable barrier membranes *in vitro*. *Clinical Oral Implants Research* **10**: 445–452.

Taguchi, Y., Amizuka, N., Nakadate, M., Ohnishi, H., Fujii, N., Oda, K., Nomura, S. & Maeda, T. (2005) A histological evaluation for guided bone regeneration induced by a collagenous membrane. *Biomaterials* **26**: 6158–6166.

Takata, T., Wang, H.L. & Miyauchi, M. (2001a) Attachment, proliferation and differentiation of periodontal ligament cells on various guided tissue regeneration membranes. *Journal of Periodontal Research* **36**: 322–327.

Takata, T., Wang, H.L. & Miyauchi, M. (2001b) Migration of osteoblastic cells on various guided bone regeneration membranes. *Clinical Oral Implants Research* **12**: 332–338.

Terheyden, H., Knak, C., Jepsen, S., Palmie, S. & Rueger, D.R. (2001a) Mandibular reconstruction with a prefabricated vascularized bone graft using recombinant human osteogenic protein-1: an experimental study in miniature pigs. Part I: prefabrication. *International Journal of Oral and Maxillofacial Surgery* **30**: 373–379.

Terheyden, H., Warnke, P., Dunsche, A., Jepsen, S., Brenner, W., Palmie, S., Toth, C. & Rueger, D.R. (2001b) Mandibular reconstruction with prefabricated vascularized bone grafts using recombinant human osteogenic protein-1: an experimental study in miniature pigs. Part II: transplantation. *International Journal of Oral and Maxillofacial Surgery* **30**: 469–478.

Vogelin, E., Jones, N.F., Huang, J.I., Brekke, J.H. & Lieberman, J.R. (2005) Healing of a critical-sized defect in the rat femur with use of a vascularized periosteal flap, a biodegradable matrix, and bone morphogenetic protein. *Journal of Bone and Joint Surgery, TheAmerican Volume* **87**: 1323–1331.

Warnke, P.H., Springer, I.N., Acil, Y., Julga, G., Wiltfang, J., Ludwig, K., Russo, P.A., Sherry, E., Sivananthan, S., Hedderich, J. & Terheyden, H. (2006) The mechanical integrity of *in vivo* engineered heterotopic bone. *Biomaterials* **27**: 1081–1087.

Warnke, P.H., Springer, I.N., Wiltfang, J., Acil, Y., Eufinger, H., Wehmoller, M., Russo, P.A., Bolte, H., Sherry, E., Behrens, E. & Terheyden, H. (2004) Growth and transplantation of a custom vascularised bone graft in a man. *Lancet* **364**: 766–770.

Wiltfang, J., Merten, H.A. & Wiltfang, J. (1996) Ectopic bone formation with the help of growth factor bFGF. *Journal of Craniomaxillofacial Surgery* **24**: 300–304.

Yang, B., Ludwig, K., Adelung, R. & Kern, M. (2006) Micro-tensile bond strength of three luting resins to human regional dentin. *Dental Materials* **22**: 45–56.

Zhang, X., Awad, H.A., O'Keefe, R.J., Guldberg, R.E. & Schwarz, E.M. (2008) A perspective: engineering periosteum for structural bone graft healing. *Clinical Orthopaedics and Related Research* **466**: 1777–1787.

© 2009 John Wiley & Sons A/S