Platelet-rich fibrin membranes as scaffolds for periosteal tissue engineering

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Abstract
Objectives: Platelet-rich fibrin (PRF)-based membranes have been used for covering alveolar ridge augmentation side in several in vivo studies. Few in vitro studies on PRF and no studies using human periosteal cells for tissue engineering have been published. The aim is a comparison of PRF with the commonly used collagen membrane Bio-Gide® as scaffolds for periosteal tissue engineering.

Material and methods: Human periosteal cells were seeded on membrane pieces (collagen [Bio-Gide®] and PRF) at a density of 10^4 cells/well. Cell vitality was assessed by fluorescein diacetate (FDA) and propidium iodide (PI) staining, biocompatibility with the lactate dehydrogenase (LDH) test and proliferation level with the MTT, WST and BrdU tests and scanning electron microscopy (SEM).

Results: PRF membranes showed slightly inferior biocompatibility, as shown by the LDH test. The metabolic activity measured by the MTT and WST tests was higher for PRF than for collagen (BioGide®). The proliferation level as measured by the BrdU test (quantitative) and SEM examinations (qualitative) revealed higher values for PRF.

Conclusion: PRF appears to be superior to collagen (Bio-Gide®) as a scaffold for human periosteal cell proliferation, PRF membranes are suitable for in vitro cultivation of periosteal cells for bone tissue engineering.

After tooth loss, the reconstruction of alveolar ridge defects is still a challenge. The aim is the cultivation of bone grafts to reconstruct the jaw bone so that dental implants can be placed. Thus far, vital autograft has been the gold standard for repair of large bone defects, because of its ability to stimulate new bone formation by recruitment of mesenchymal stem cells (MSC). Kostopoulos & Karring (1995) and other research groups [Stevenson 1999] showed that the periosteum and especially the MSC contained in the cambium layer play a crucial role in bone autograft healing by differentiation into bone cells. Based on these findings, periosteal cells are currently a prime focus in bone tissue engineering. A main problem in bone transplantation research is the application of these cells in vivo.

One of the pioneers of tissue regeneration was Buser et al. (1993), who introduced the term ‘guided bone regeneration (GBR)’ to describe the support of bone augmentation by a barrier membrane. Many membrane systems, both non-resorbable and resorbable, have been investigated in the past few years. Until now, bioresorbable membranes have been preferred because they permit a single-stage
procedure without removal and less bacterial colonization [Rothamel et al. 2005]. Hence, a main goal in tissue engineering is to create a resorbable membrane system to grow periosteal cells for bone tissue engineering. In general, these membranes could be of synthetic origin or derived from natural materials like collagen (BioGide®), a membrane composed of porcine type I and type III collagen fibers. Arnold et al. [2002] revealed that a synthetic resorbable composite of PGA polymer (polyglycolic acid-co-lactic acid), TCP and fibrin led to enhanced proliferation and osteogenic differentiation of periosteal cells in vitro. It could be shown that periosteal cell matrix composites can be spatially transferred with fibrin glue from culture dishes to recipient sites in animal models without loss of viability of cultured tissue [Isogai et al. 2000].

Thus, the use of vital fibrin as an autologous scaffold for periosteal cell or stem cell transplantation and consequently for bone tissue engineering is an obvious option. It is biocompatible, bioresorbable, and plays an essential role in wound repair, not only for hemostasis but also provides a matrix for migration of tissue-forming cells like fibroblasts and endothelial cells, which are involved in angiogenesis and that are responsible for remodeling of the new tissue. In the normal wound-healing process, platelets are trapped within the fibrin matrix and are subsequently activated so that growth factors like platelet-derived growth factor [PDGF], transforming growth factor-β [TGF-β] and insulin-like growth factor I [IGF-I] are set free [Schliephake 2002], which could stimulate the mitogenic response of the periosteum during bone repair [Gruber et al. 2003].

The essence of platelet-rich fibrin (PRF) modified by Choukroun and colleagues [Dohan et al. 2006a] is a fibrin matrix in which the platelet cytokines and cells are trapped and may be delivered after a certain time [Mosesson 2005]. Although there are several studies on the use of Choukroun’s PRF in vivo [Choukroun et al. 2006a, 2006b], there are relatively few studies concerning cell culture on PRF in vitro [Choukroun et al. 2007; Dohan et al. 2007; Gassling et al. 2009]. It could be shown that PRF can serve as a resorbable membrane that can be used in preprosthetic surgery as well as in implantology to cover bone augmentation sites [Choukroun et al. 2006b]. These, and other studies involving other PRF preparations [Lundquist et al. 2008], have concentrated on the effect of PRF added to two-dimensional cell cultures on cell culture polystyrene and not on direct culture of cells on PRF. To the authors’ best knowledge, there are no in vitro studies comparing PRF with other membranes (e.g., the commonly used collagen membrane, BioGide®) or involving the use of periosteal cells that are commonly used in bone tissue engineering applications.

With a view to developing new scaffold materials, in this study, biocompatibility and ability to support and promote the proliferation of human periosteal cell proliferation was measured for the first time on PRF membranes produced using the method of Choukroun and colleagues [Dohan et al. 2006a] and compared with the conventional collagen membrane BioGide® in vitro.

Material and methods

Isolation and cultivation of cells from the periosteum

Human periosteum biopsy was harvested from one patient [male, 24 years old] during the course of oral surgery (wisdom tooth removal). The study [AZ 417/07] was approved by the Ethics Commission of the Christian-Albrechts-University of Kiel, Germany. The cells were cultured using an osteogenic medium consisting of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 IE penicillin/ml, 100 μg streptomycin/ml and 1 mmol/l ascorbic acid at 37°C with 5% CO₂. Cell seeding was performed after the second passage. During passageing, cells were detached from 75 cm² cell culture flasks using 5 ml of a 0.05% trypsin/0.02% EDTA solution in phosphate-buffered saline [PBS]. After a 1:1 dilution of the cell suspension with DMEM containing 10% FCS and centrifugation at 3000 g for 3 min, cells were resuspended in DMEM containing 10% FCS, counted and reseeded at a density of 10⁵ cells/75 cm² cell culture flask. Cells were cultured in the same medium used for cell seeding in a humidified atmosphere with 5% CO₂ at 37°C. Medium change took place every 3 days.

Membranes

BioGide® [Geistlich AG, Baden-Baden, Germany] (porcine collagen types I and III, non-crosslinked) and PRF produced using the method of Choukroun and colleagues [Dohan et al. 2006a] served as scaffolds for the cultivation of human periosteum cells.

PRF membranes were produced in the following way:

Briefly, one healthy study participant [male, 35 years] donated 40 ml of his whole blood in 10 ml tubes without an anticoagulant [Vacuette 455092, Greiner Bio-One, Frickenhausen, Germany]. Ethical approval [AZ 118/07] was granted by the Ethics Commission, Christian-Albrechts-University of Kiel, Germany. Blood samples were immediately centrifuged for 12 min at 2700 r.p.m. (approximately 400g). After activation of the coagulation cascade by contact of blood platelets with the tube walls, a fibrin clot was obtained in the middle between the plasma at the top and red blood cells at the bottom of the receptacle. PRF clot was separated from the red blood cells using a sterile syringe and scissors and then transferred onto a sterile compress. A stable fibrin membrane was obtained by squeezing serum out of the PRF clot [Fig. 1a–d].

Both membranes, BioGide® and PRF, were cut into quadratic pieces of side length 7 mm and placed in 24-well cell culture plates [Nunc, GmbH, Langenelshöld, Germany]. Cells were seeded on membrane pieces at a density of 10⁴ 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% CO₂ 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 fluorescein diacetate [FDA] and propidium iodide [PI] staining. Staining was performed on cells cultured in an eluate from membranes after incubation for 10 min, 1 or 24 h in a serum-free cell culture medium. 5 × 10⁴ cells in cell culture medium with 10% FCS were seeded on membrane pieces in 2000 μl of the same medium used for cell seeding in a humidified atmosphere with 5% CO₂ at 37°C. After 1 day of culture, 200 μl eluate from membranes immersed in a serum-free cell culture medium for 10 min, 1 or 24 h was added to cells. After a 24 h incubation at 37°C and
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 and proliferation tests

Lactate dehydrogenase (LDH) and 5-bromo-2-deoxyuridine (BrdU), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) and WST tests

The LDH test 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 x 10^4 cells/well. After a 24 h culture in a humidified atmosphere with 5% CO₂ 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 850 μl eluate per well remaining in the cell culture plate was removed and replaced with 100 μl DMEM containing 10% FCS, 100 IU penicillin/ml, 100 μg streptomycin/ml and 1 mmol/l ascorbic acid. After 5 days of incubation, proliferation was measured with the help of a BrdU Cell Proliferation ELISA kit [Roche Diagnostics, Cat. No. 1164729001]. This method is based on the incorporation of BrdU instead of thymidine into newly synthesized DNA of proliferating cells. Absorbance was measured at 450 nm.

As mentioned previously, membrane pieces were seeded at a density of 10⁴ 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, Cat. No. 11646807001]. The evaluation of cell proliferation is based on the cleavage of tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. 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% CO₂ 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 10⁴ cells/well served as controls.

Scanning electron microscopy (SEM) examinations

SEM investigations were carried out 1 week after cell seeding using an XL30CP device [Philips 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 3 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

Each sample value represents one well. Absorbance values of the tests were related to the mean results obtained without membrane materials. All values of the LDH test were divided by 0.07 [mean of low control], the values of the MTT test by 0.26, the WST results by 1.22 and the BrdU values by 1.52. These results were analysed by ANOVAs for each test with the factors material [PRF, BioGide] and time [10 min, 1 and 24 h]. Least squared means and 95% confidence intervals are presented in the text and figures.
Results

Assessment of cell vitality on membranes
After 10 min, 1 and 24 h, all samples showed viable periosteal cells. The pronounced green color of the cells due to FDA staining demonstrated their vitality on both membranes, whereas the absence of a red color despite PI staining indicated that no cells died as a result of eluate from membranes. These results are illustrated in Figs 2 and 3.

Biocompatibility tests
In the case of the LDH test, cytotoxicity after treatment with an eluate from the collagen membrane after incubation in a serum-free cell culture medium was similar to the low control (zero cytotoxicity). In the case of PRF, the LDH test results were around five times as high as the low control \( (P<0.0001) \); see Fig. 4 and Table 1a). The values were especially high after 1 h \( (5.06\text{ compared with 1.47 after 10 min and 2.76 after 24 h}; P<0.0001) \). 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. In the case of the MTT test, metabolic activity on both membranes was slightly higher than the control \( (>1) \) and the materials showed different results \( (P=0.0007); \) see Fig. 5 and Table 1b). No differences were seen for the time effect \( (P=0.95) \) (results not shown). The WST test revealed values of around 0.35 for collagen and 2.21 for PRF membranes \( (P<0.0001); \) see Fig. 6 and Table 1c). The high values of the PRF group indicate a high metabolic activity. BrdU tests show cell proliferation by incorporation of BrdU during DNA synthesis. The results were between 0.89 (collagen) and 1 (PRF) \( (P<0.0001); \) see Fig. 7 and Table 1d).

SEM investigations of cell morphology on membranes
Periosteal cells formed layers covering the surfaces of both membranes 7 days after seeding. The close-up images in Fig. 8 show that both membranes were almost completely covered by cells, which had an elongated morphology with numerous cell pseudopodia, suggesting good biocompatibility.

Discussion

Tooth loss often leads to alveolar ridge atrophy. For stable insertion of dental implants, adequate jaw bone is an essential precondition. Thus, the primary aim in implantology is the regeneration of lost bone tissue, e.g., through scaffold–cell constructs grown outside the body (Vacanti & Vacanti 2000). The perfect scaffold material for cultivation of periosteal cells or stem cells has not yet been found.

Membranes have been used for many years in guided tissue regeneration (GTR)
membrane was slightly higher than the control. LSM, least squared mean, CI, confidence interval.

Table 1. Mean, SDs and number of samples for the two treatment groups for the three time points

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Time</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
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<tr>
<td>Collagen</td>
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<td>(c) WST test</td>
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<tr>
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<td>(d) BrdU test</td>
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LDH, lactate dehydrogenase; BrdU, 5-bromo-2-deoxyuridine.

showed that for mouse osteoprogenitor cells, collagen is a favorable scaffold for osteoblastic cell proliferation (Takata et al. 2001).

The rationale for a cell culturing system based on fibrinogen is due to its ability to enhance cell attachment (Gorodetsky et al. 1998), proliferation (Sporn et al. 1993) and differentiation (Huang et al. 2002). Several publications have judged fibrin to be a suitable scaffold material for colonization of human MSC (HMSC) (Catelas et al. 2006; Trombi et al. 2008) and HMSC are able to adhere, spread and proliferate, depending on different fibrinogen concentrations (Bensaid et al. 2003). Perka et al. (2001) developed a bioresorbable alginate-fibrin vehicle that ensured an initial cell proliferation and differentiation to establish a stable matrix structure for transplantation of different cell types like periosteal cells and chondrocytes.

There may be varying abilities of fibrin- and collagen-based substrates to support proliferation and topographical differences. Unfortunately, there are only a few studies in the literature that deal with this context. The equation of fibroblast and keratinocyte proliferation in collagen and fibrin gels revealed no differences after 2 days of cultivation (Hojo et al. 2003). Another publication revealed superior growth of placental trophoblasts on collagen I-coated cell culture plastic compared with fibrin coatings after 24 and 72 h (Farmer & Nelson 1992). Both these studies may suggest that the superior proliferation on PRF is not due to fibrin. However, it is known that smooth surfaces promote proliferation better than rough surfaces. The surface of PRF membranes is considerably smoother than that of the bone-friendly side of collagen (BioGide®) [Fig. 8], which may partly explain the above-mentioned higher proliferation on PRF.

Another possible explanation for higher cell proliferation on PRF membranes compared with collagen (BioGide®), as shown by the results of the BrdU test [Fig. 7] and SEM investigation [Fig. 8], is the above-mentioned release of growth factors from platelets in PRF, which influences proliferation. The relationships between platelet-released supernatants and mitogenic activity of periosteal cells were shown by Gruber et al. (2003). They could show that factors set free from the activated platelets
like PDGF and basic fibroblast growth factor (bFGF) increased the number of proliferating periosteum cells. Both Choukroun’s PRF (Dohan et al. 2006c; Gassling et al. 2009) and other PRF preparations (Lundquist et al. 2008) are known to contain platelets and thus growth factors such as PDGF, TGF-β and IGF-I, which can promote cell proliferation.

Stem cells reside in a unique microenvironment composed of extracellular matrix and resident cells, also known as a niche (Dawson et al. 2008). Such vital microenvironments composed of extracellular matrix and entrapped platelets containing cytokines were found in PRF, which was first described by Choukroun and colleagues (Dohan et al. 2006a). The good results for PRF suggest that one possible explanation is the more physiological natural and progressive polymerization during centrifugation of PRF like nullifies by Choukroun and colleagues (Dohan et al. 2006b). The fibrin network thus formed presents a particularly homogeneous three-dimensional organization, even more highly coherent than natural fibrin clots. Moreover, a progressive polymerization mode signifies increased incorporation of the circulating cytokines into the fibrin meshes (intrinsic cytokines). In contrast, in collagen membranes there is no vital microenvironment. BioGide® is composed of porcine collagen fibers without growth factors. Despite the fact that collagen fibers are the major components in extracellular bone matrix that mediate cell–matrix attachment of osteogenic cells, the lack of bone-specific proteins leads to a purely physical scaffold nature of BioGide®. Until now, there is only a slight assumption that collagen fibers may trap osteoinductive factors from the bone (Koda & Bernfield 1984).

Conclusion

Here, for the first time, we could show that PRF is a suitable scaffold for breeding human periosteal cells in vitro, which may be suitable for bone tissue engineering applications. Nevertheless, both systems, PRF and collagen (BioGide®), seem to be suitable as scaffold materials for the generation of periosteum layers. However, it should be borne in mind that the vital property of PRF is a benefit not only because of its biocompatibility but also because its use results in lower costs.

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Gassling et al Scaffolds for periosteal tissue engineering

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