CLINICAL ORAL IMPLANTS RESEARCH

Volker Gassling
Jürgen Hedderich
Yahya Açil
Nicolai Purcz
Jörg Wiltfang
Timothy Douglas

Comparison of platelet rich fibrin and collagen as osteoblast-seeded scaffolds for bone tissue engineering applications

Authors' affiliations:
Volker Gassling, Yahya Açil, Nicolai Purcz, Jörg Wiltfang, Department of Oral and Maxillofacial Surgery, University of Schleswig-Holstein, Kiel, Germany
Jürgen Hedderich, Institute of Medical Informatics and Statistics, University Hospital of Schleswig-Holstein, Kiel, Germany
Timothy Douglas, Department of Biomaterials, Radboud University Medical Center, Nijmegen, Nijmegen, The Netherlands

Corresponding author:
Volker Gassling
Department of Oral and Maxillofacial Surgery, University of Schleswig-Holstein, Campus Kiel, Arnold-Heller-Straße 3, Haus 26, 24105 Kiel, Germany
Tel.: +49 431 597 2791
Fax: +49 431 597 4084
e-mail: gassling@mkg.uni-kiel.de

Key words: bone tissue engineering, collagen membrane, human osteoblast cells, platelet-rich-fibrin membrane

Abstract

Objectives: The loss of jaw bone caused by different kinds of pathologies leads to dysfunction and reduced quality of life in affected patients. Thus, the pivotal goal in bone tissue engineering is to reconstruct these defects. The essential precondition for new tissue generation is an extracellular matrix which acts as a scaffold so that cells can migrate, differentiate, and proliferate. Fibrin, a biopolymer responsible for blood clot formation, has been shown to be suitable for tissue engineering applications. The aim of the present study is a comparison of platelet rich fibrin (PRF) with the commonly used collagen membrane BioGide® as a scaffold for human osteoblast cell seeding for bone tissue engineering.

Material and methods: Human osteoblasts were cultured with eluates from PRF ($n = 7$) and BioGide® ($n = 8$) membranes incubated in serum-free cell culture medium. Vitality of these cells was assessed by fluorescein diacetate and propidium iodide staining, biocompatibility with the lactate dehydrogenase test and proliferation levels with the MTT ([3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide]), and BrdU (5-bromo-2-deoxyuridine) tests. In addition, human osteoblasts were seeded on both membrane systems and cell growth was compared by the water soluble tetrazolium (WST-1) (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) test and scanning electron microscopy (SEM). Osteoblastic differentiation was assessed by alkaline phosphatase activity measured by ELISA in the supernatant of osteoblasts cultivated on PRF membranes ($n = 10$), PRF clots ($n = 10$), and BioGide® membranes ($n = 10$).

Results: Lactate dehydrogenase test values were higher for PRF compared to BioGide®. The BrdU test showed superior cell growth after cultivation in eluate from PRF than in eluate from BioGide®. The WST-1 assay demonstrated superior cell proliferation on PRF than on BioGide®. SEM revealed osteoblast colonization of both membranes. Cultivation of osteoblasts on PRF membranes and PRF clots showed significantly higher alkaline phosphatase activity than on BioGide® membranes.

Conclusion: Metabolic activity and proliferation of human osteoblast cells *in vitro* were supported to a significant higher extent by eluates from PRF membranes. Both membranes are suitable as scaffolds for cultivation of human osteoblast cells *in vitro*; proliferation was significant higher on PRF membranes and on PRF clot than on BioGide® membranes.

The pivotal goal in bone tissue engineering is to regenerate defects with scaffold-cell constructs grown outside the body and subsequently to transplant them into patients (Vacanti & Vacanti 2000). Up to now a wide range of synthetic and natural materials have been considered to be suitable for bone tissue engineering, although an ideal scaffold material has not yet been found (Frohlich et al. 2008). There is still controversy regarding the importance of structure and composition of these materials and there are a vast number of issues which affect the success of tissue engineering, of which most relate to the cell-scaffold interaction. The ideal scaffold material has to mimic physiological conditions so that osteogenic cells can migrate, proliferate, and differentiate and thus form new bone.

Until now the gold standard for reconstruction of large jaw bone defects has been autologous bone transplants due to their osteoinductive and osteoconductive properties. The main disadvantages are the patient's morbidity, and size restriction of bone trans-

Date:
Accepted 25 August 2011

To cite this article:
Gassling V, Hedderich J, Açil Y, Purcz N, Wiltfang J, Douglas T. Comparison of platelet rich fibrin and collagen as osteoblast-seeded scaffolds for bone tissue engineering applications.
Clin. Oral Impl. Res. **24,** 2013, 320–328
doi: 10.1111/j.1600-0501.2011.02333.x

plants. However, knowledge of bone graft healing processes may be crucial for new insights in bone tissue engineering research. The insertion of bone graft transplants, both cancellous and cortical, involves a series of sequential cell events which ideally leads to incorporation into the host skeleton (Burchardt 1983). In brief, the healing process could be divided in three steps, whereby the first phase is characterized by blood clot formation, inflammatory response, and fibroblast-like mesenchymal cell chemotaxis and thus initiation of the bone induction cascade. A few days after implantation of bone matrix the occurrence of chondroblasts indicates the beginning of the differentiation phase (second phase). These processes are concomitant by cartilage maturation and result in endochondral bone formation. Vascular invasion marks the final phase of bone induction. The bone graft healing is completed by the appearance of osteoblasts and new bone formation (third phase). Finally, the bone remodeling process is characterized by the interaction of osteoblasts and osteoclasts (Zipfel et al. 2003). In summary, bone graft healing mainly depends on the interaction of cells, extracellular matrix (ECM) (Damsky 1999), vascularization (Santos & Reis 2010), and cytokines (Schliephake 2002) and each of them should be considered when developing new scaffold materials for bone tissue engineering.

One of the first and essential steps in bone graft healing process is the aforementioned blood clot formation primarily mediated by fibrinogen and fibrin polymerization. This process leads to a wide range of biological effects like growth factor binding, and interaction with cells like platelets, fibroblasts, leukocytes, endothelial cells, and circulating stem cells. Thus, the rationale for the biopolymer fibrin as a scaffold for bone tissue engineering is an obvious option and there are many hints in international literature which support this hypothesis. Several publications have judged fibrin to be a suitable scaffold material for colonization of human mesenchymal stem cells (HMSC) (Catelas et al. 2006; Trombi et al. 2008) and it could be shown that HMSC are able to adhere, spread, and proliferate, depending on different fibrinogen concentrations (Bensaid et al. 2003). Furthermore, 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).

Commonly used forms of fibrin scaffolds in tissue engineering are fibrin microbeads, fibrin glue, and fibrin hydrogels (Ahmed et al. 2008). Platelet rich fibrin (PRF), first described by Choukroun et al. (2001), is an autologous blood substrate. Its essence is a fibrin matrix in which the platelet cytokines and cells are trapped and may be delivered after a certain time (Mosesson 2005). These cytokines could be shown to stimulate the mitogenic response of the periosteum during early stage of bone repair (Gruber et al. 2003) and in generally are strongly associated with the bone healing process (Lind 1998). It has been known that osteoblasts are essential for synthesis and mineralization of extracellular bone matrix and thus the cultivation of these cells on different kinds of scaffold materials seems to be of special interest for bone tissue engineering. Until now, there has only been one study in international literature comparing PRF with the commonly used collagen membrane BioGide® as a scaffold for periosteal tissue engineering (Gassling et al. 2010) and to our best knowledge there are no studies which compare cell growth of human osteoblasts on PRF and collagen membranes.

The present study was performed with a view to developing new scaffold materials for bone tissue engineering. Here for the first time PRF and collagen membranes were compared as scaffolds for human osteoblast cell cultivation. The objectives of the present study were as follows: (i) to compare the biocompatibility of both membrane systems, collagen (BioGide®) and PRF; (ii) to evaluate human osteoblast cell vitality and proliferation when cultured with eluates from PRF and collagen membranes (BioGide®); (iii) to determine the proliferation of human osteoblast cells when seeded on both membrane systems; and (iv) to measure osteoblast differentiation by alkaline phosphatase (ALP) activity measured in the supernatant of osteoblasts cultivated on PRF membranes, PRF clots, and BioGide® membranes, respectively.

Material and methods

Isolation and cultivation of cells

It is known that the phenotype of human osteoblasts differs depending on their skeletal site of origin and thus for the present study only cells from oral sites were isolated (Kasperk et al. 1995). Cells were harvested from one male patient (39 years old) during the course of oral surgery (wisdom tooth removal). The study was approved by the ethics board of the Christian-Albrechts-University of Kiel, Germany (Ref: AZ 417/07). The human osteoblasts were cultivated using a medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 100 IE penicillin/ml, 100 µg streptomycin/ml, 1 mmol/l ascorbic acid and 100 nmol/l dexamethasone 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/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 200 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 flasks. 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.

Cell characterization

The phenotype of human osteoblasts was confirmed by detection of osteocalcin production. Cells were seeded on eight-well objectives and incubated with a monoclonal antibody directed against osteocalcin (Abcam, Cambridge, UK). The control-cells were incubated with 1% bovine serum albumin (Sigma-Aldrich GmbH, Hamburg, Germany). After incubation with anti-osteocalcin antibody, the cells were washed and incubated with enzyme-conjugated secondary antibody (Dako GmbH, Hamburg, Germany). The enzymatic detection was performed with horseradish peroxidase (Dako GmbH) and counterstaining was carried out with hematoxyline eosin (Merck, Darmstadt, Germany).

Membranes

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

PRF membranes were produced in the following way:

Briefly, seven healthy study participants donated 40 ml of whole blood in 10 ml tubes without anticoagulant (Vacuette 455092; Greiner Bio-One GmbH, Frickenhausen, Germany). Ethical approval (Ref: AZ 118/07) was issued by the Ethics Commission, Christian-Albrechts-University of Kiel, Germany. Blood samples were immediately centrifuged for 12 min at 400 *g*. 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. The 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 (see Fig. 1a–d).

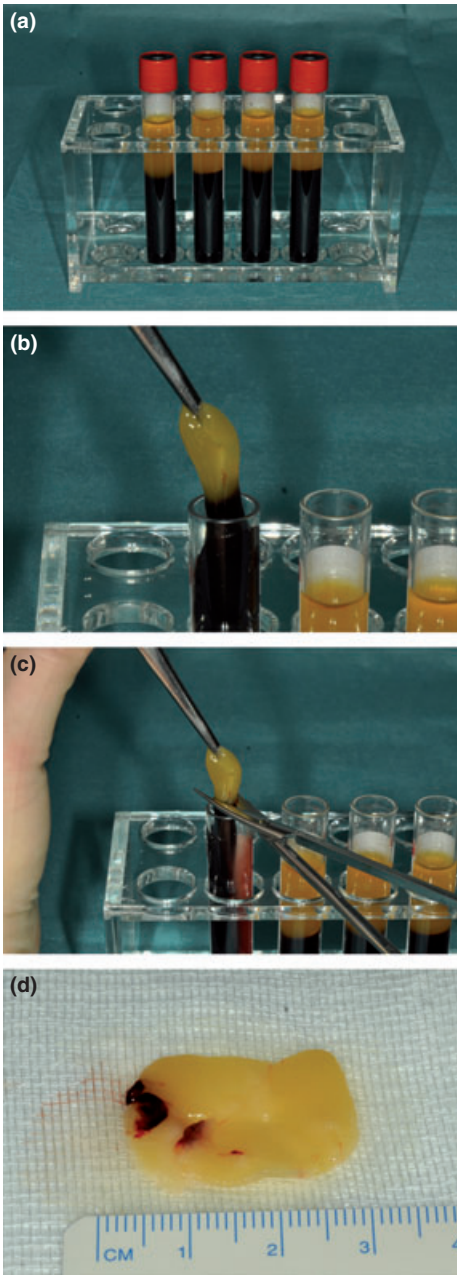

Fig. 1. Photographs of platelet-rich-fibrin (PRF) membrane preparation. (a) Fibrin clot in the middle of the tube, between the red blood cells at the bottom and platelet poor plasma at the top; (b) stable fibrin clot adherent to the red blood cells; (c) separation of PRF clot from the red blood cells using a sterile syringe and scissors; (d) resistant autologous PRF membrane after squeezing out serum.

Eluate extraction for further analysis

Both membranes, PRF and BioGide®, were cut into quadratic pieces of side length 7 mm, and then for each membrane three pieces were placed in a 24-well cell culture plate (Nunc GmbH, Langenselbold, Germany). Each membrane was incubated with 2.5 ml DMEM medium without FCS for 10 min, 1 and 24 h and then the eluate was removed and stored for further analysis at 4° C.

Assessment of cell vitality

Cell vitality was assessed by fluorescein diacetate (FDA) and propidium iodide (PI) staining. 1×10^4 cells in cell culture medium with 10% FCS were seeded on 8-well objectives (Lab-TEKII Chamber Slide w/cover RS glass slide; Nalge Nunc International, Roskilde, Denmark). After 1 day of culture, 200 µl eluates from membranes immersed in serum-free cell culture medium for 10 min, 1 or 24 h was added to cells. After 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, slides 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 BrdU (5-bromo-2-deoxyuridine) test

The LDH test can show cell death and lysis. Cells were seeded in 96-well cell culture plates (Nunc GmbH) in 100 µl DMEM with 10% FCS 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 24-h incubation, 100 µl of the eluate was transferred to another 96-well cell culture plate. Extracellular LDH activity was measured with the help of an LDH detection kit (Catalogue No. 11644793001; Roche Diagnostics, Mannheim, Germany). Absorbance was measured at 490 nm. Calibration curves of 5-0.16 × 10^3 cells/well served as standards. The remaining 50 µl eluate per well remaining in the cell culture plate was removed and replaced with 100 µl DMEM containing 10% FCS, 100 IE 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 (Catalogue No. 11647229001; Roche Diagnostics). 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.

MTT ([3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium-bromide]) test

Cell culturing and measurement was performed according to the aforementioned procedure. After 24-h incubation with an eluate from the membranes proliferation was assessed with the aid of an MTT Cell Proliferation Kit (Catalogue No. 11465007001; Roche Diagnostics). The detection of cell vitality is based on the reduction of a yellow colored dye (MTT) to blue-violet Formazan according to the method of Mosmann (1983). Absorbance was measured at 550 nm.

Water soluble tetrazolium (WST-1) (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) test

Cells were seeded on the aforementioned membrane pieces at a density of 1×10^4 cells/well after removal of eluate. Cells were cultured 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. After 7 days of culture proliferation was assessed with the aid of a Cell Proliferation Reagent WST-1 (Catalogue No. 116446807001; Roche Diagnostics, Mannheim, Germany). 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 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 460 nm. Cells cultured in wells without membrane pieces at a density of 10^4 cells/well served as controls.

A general schematic overview of the experiments is given in Fig. 2, part I.

Scanning electron microscopy (SEM) examinations

Scanning electron microscopy investigations were carried out 1 week after cell seeding

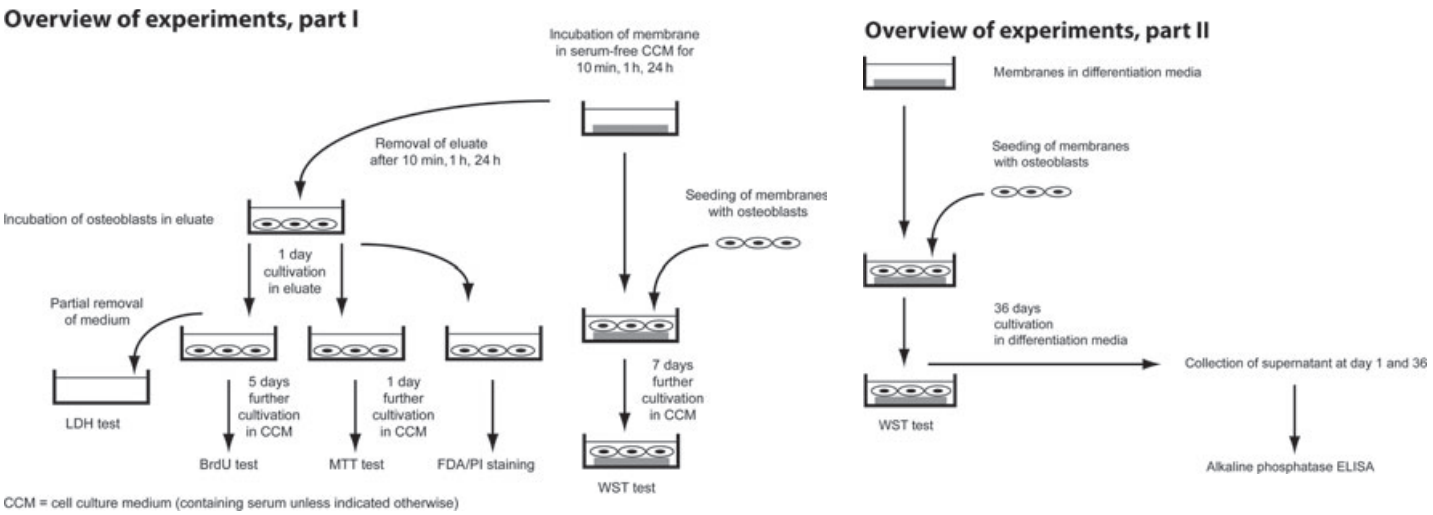

Fig. 2. General schematic overview of experiments performed in this study; part I: lactate dehydrogenase (LDH) test, MTT ([3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide]) test, BrdU (5-bromo-2-deoxyuridine) test, fluorescein diacetate/propidium iodide (FDA/PI) staining, water soluble tetrazolium (WST-1) (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) test; part II: alkaline phosphatase ELISA.

(1×10^4 cells/well) using an XL30CP device (Phillips Electron Optics GmbH, Kassel, Germany) operating at 10–25 kV, as used by Yang et al. (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).

ALP activity

The 24-well plates were prepared with 900 µl of differentiation media (DMEM) with 10% FCS, 100 nmol dexamethasone, 10 µmol beta-glycerol phosphate, 52 mg/l ascorbin-2-phosphate, and 230 mg/l Ca-chloride. The PRF clot was obtained from 10 ml whole blood. Ten PRF clots were placed in a 24-well plate. Another 10 PRF clots were compressed to obtain membranes as described above and then were placed in another 24-well plate. BioGide® membranes were cut into 10 square pieces of size 8×8 mm and placed in another 24-well plate. Human osteoblasts (1×10^4 cells in 100 µl differentiation media) were seeded on the PRF membrane, PRF clot, and BioGide® membrane, respectively. The supernatant was collected on day 1 and day 36. Cell culture media were changed weekly. ALP activity was measured with an ELISA kit (Uscn Life Science Inc., Wuhan, China).

A general schematic overview of the experiments is given in Fig. 2, part II.

Statistical evaluation

Statistical analysis was performed using SPSS statistical software (Version V.18) and in the R-Software especially the F1_LD_F1.R macro from Mahbub Latif (http://www.ams.med.uni-goettingen.de/amsneu/sasmakr-de.shtml) was used to analyze longitudinal data.

Each sample (value) represents one well. LDH was transformed to percentage values (%) by the distance to the low value related to the observed range between high-low values. All other tests (MTT, WST-1, and BrdU) were transformed to percentage values (%) in relation to control measurements. All results are summarized by means and standard deviations as well as median and quartile values in Table 1. Median values are shown together with individual measurements in the Figs 5–8. There was no evidence against the assumption of normal distribution based on Kolmogorov–Smirnov test. With respect to a low power of this test and different variances the non-parametric longitudinal analysis from Brunner and Langer (Brunner et al. 2002) was used for the evaluation of suspected effects from different membranes and time points on the extinction. *P*-values are summarized in Table 2. The level of significance was chosen for all tests at 0.05. The effect of different membrane types on the alteration of ALP activity between day 1 and day 36 were analyzed with a rank based Kruskal–Wallis test, followed by a multiple comparison procedure (Dunn test).

Results

Assessment of cell vitality on membranes

After 10 min, 1 and 24 h, all samples showed viable cells. The pronounced green color of the cells due to FDA staining demonstrated their vitality on both membranes, while the absence of a red color despite PI staining indicated that no cells died as a result of eluates from both membranes. These results are illustrated in Figs 3 and 4.

Biocompatibility tests

In the LDH test, cytotoxicity after treatment with an eluate from the collagen membrane (BioGide®) after incubation in a serum-free cell culture medium showed very low cytotoxicity. In the case of PRF, there is a significant increase in LDH signal with increasing eluation time, i.e. duration of membrane incubation in serum-free cell culture medium compared to BioGide® (see Fig. 5).

The MTT test gives a measure of cellular metabolic activity dependent on living cells, proliferation, viability, and cytotoxicity. The results showed a significant decrease of PRF signal compared to BioGide® with increasing eluation time (see Fig. 6).

At a later time point than the MTT test, the WST-1 test indicates the metabolic activity of cells. In addition, the WST-1 test is performed using cells directly seeded on scaffolds, not cells cultured in eluate from scaffolds. In the WST-1 assay the test values for

Table 1. **Descriptive statistics from BioGide® (n = 8) and PRF (n = 7) of relative extinction in LDH, MTT, WST-1 and BrdU assays at different time points (10 min, 1, and 24 h)**

Membrane type	Test	Time	Mean	SD	Min.	Max.	1. Quartile	Median	3. Quartile
BioGide (n = 8)	LDH	10 min	4.33	6.83	−2.51	18.38	−0.29	1.98	8.70
		1 h	2.10	5.79	−2.06	14.63	−1.93	−0.43	5.17
		24 h	5.53	7.51	−0.80	19.15	−0.24	2.04	12.67
	MTT	10 min	107.00	7.69	94.67	118.26	101.95	107.04	114.25
		1 h	106.35	6.55	99.12	114.63	99.41	106.45	112.61
		24 h	110.65	11.88	95.15	127.29	100.69	109.39	122.23
	WST-1	10 min	77.60	11.60	56.04	89.53	68.22	80.45	87.17
		1 h	70.76	9.95	56.40	85.81	61.59	72.00	78.36
		24 h	67.52	6.64	59.39	79.67	60.92	67.63	71.35
	BrdU	10 min	54.32	47.85	8.09	106.46	9.80	49.88	100.23
		1 h	108.14	30.32	66.80	141.61	79.93	111.84	137.53
		24 h	121.26	39.69	75.59	169.01	84.40	119.18	160.83
PRF (n = 7)	LDH	10 min	5.71	4.61	−1.91	11.37	1.85	5.63	9.66
		1 h	17.96	13.52	3.52	37.42	5.54	16.67	35.18
		24 h	32.93	17.97	10.87	63.43	19.51	25.80	44.39
	MTT	10 min	88.63	20.53	69.65	132.79	76.37	84.39	88.59
		1 h	68.14	27.55	46.88	125.83	49.63	61.41	77.83
		24 h	65.46	19.57	49.83	104.65	51.02	55.60	71.76
	WST-1	10 min	179.51	31.98	148.47	245.78	162.18	169.95	190.53
		1 h	179.17	25.46	154.85	230.42	156.62	176.42	184.30
		24 h	185.72	35.42	145.23	254.57	162.61	173.28	203.02
	BrdU	10 min	135.46	18.86	101.83	159.46	125.21	133.89	151.94
		1 h	156.05	20.15	132.59	191.60	139.37	155.59	166.24
		24 h	163.21	35.12	100.34	201.22	150.50	156.37	200.71

PRF, platelet rich fibrin; LDH, lactate dehydrogenase; MTT, [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide]; WST-1, water soluble tetrazolium (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate); BrdU, 5-bromo-2-deoxyuridine.

Table 2. ***P*-values from non-parametric longitudinal data analysis for LDH, MTT, WST-1, and BrdU assays**

Test	Membrane (A)	Time points (T)	Interaction ($A \times T$)
LDH	0.0001	0.0005	0.0045
MTT	0.0005	0.0122	0.0026
WST-1	<0.0001	0.2710	0.0397
BrdU	0.0001	0.0001	0.6067

LDH, lactate dehydrogenase; MTT, [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide]; WST-1, water soluble tetrazolium (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate); BrdU, 5-bromo-2-deoxyuridine.

PRF are at a significantly higher level than BioGide® independently of eluation time (see Fig. 7).

The BrdU tests show cell proliferation by incorporation of BrdU during DNA synthesis. In BioGide® there is a stronger increase in period of time with significantly higher mean values of PRF at all eluation times (see Fig. 8).

Descriptive statistics are shown in Table 1.

SEM investigations of cell morphology on membranes

All cells formed layers covering the surfaces of both membranes 7 days after seeding. The close-up images in Fig. 9 show that both membranes were almost completely covered by cells which formed a three-dimensional structure, suggesting good biocompatibility. More cells, forming confluent layers, could be observed on PRF.

ALP activity

The differences of ALP activity are shown in Table 3 and Fig. 10. A significantly higher increase in ALP activity was observed in the cultures on PRF membranes (11 U/l) and PRF clots (10.5 U/l) than in the cultures on BioGide® membranes (1.2 U/l). The Kruskal–Wallis test showed significant differences between the membrane types ($P < 0.001$). Based on non-parametric multiple comparisons (Dunn test) significant differences between the cultures seeded on PRF membranes and BioGide® membranes and between the cultures seeded on PRF clots and BioGide® membranes but not between the cultures seeded on PRF membranes and PRF clots ($P < 0.05$) could be shown.

Discussion

The present study was designed to evaluate human osteoblast cell vitality and proliferation when cultivated with eluates of PRF and collagen membranes (BioGide®) and to determine the proliferation of osteoblasts on both membranes. In addition the cell differentiation on PRF clots, PRF membranes, and BioGide® membranes was compared. An essential precondition for the cultivation of cells on scaffold materials are biocompatibility and adhesion, of which the latter essentially influences a cell's capacity to proliferate and differentiate. It was known that differing characteristics such as the scaffold surface substantially influence cell adhesion. The investigation of different biomaterials revealed a higher adhesion and migration of bone cells on smooth than on rough surfaces (Naji & Harmand 1990; Anselme et al. 2000). However, there are other investigations which revealed higher proliferation for osteoblast cells on rough surfaces (Kunzler et al. 2007). These varying results may be partially caused by different experimental conditions such as for e.g. the maturation state of the osteoblast cells used. It could be shown that osteoblastic cells respond in a differential manner to changes in the surface roughness depending on their maturation state (Lohmann et al. 2000). Accordingly to the evaluation of SEM investigations in the present study, surfaces of PRF membranes were smoother in comparison to BioGide®, whereby PRF membrane was almost completely covered by cells (see Fig. 9).

Besides the pure physical roughness there are surface characteristics such as topography, chemistry or surface energy which seem to be of importance for adhesion of osteo-

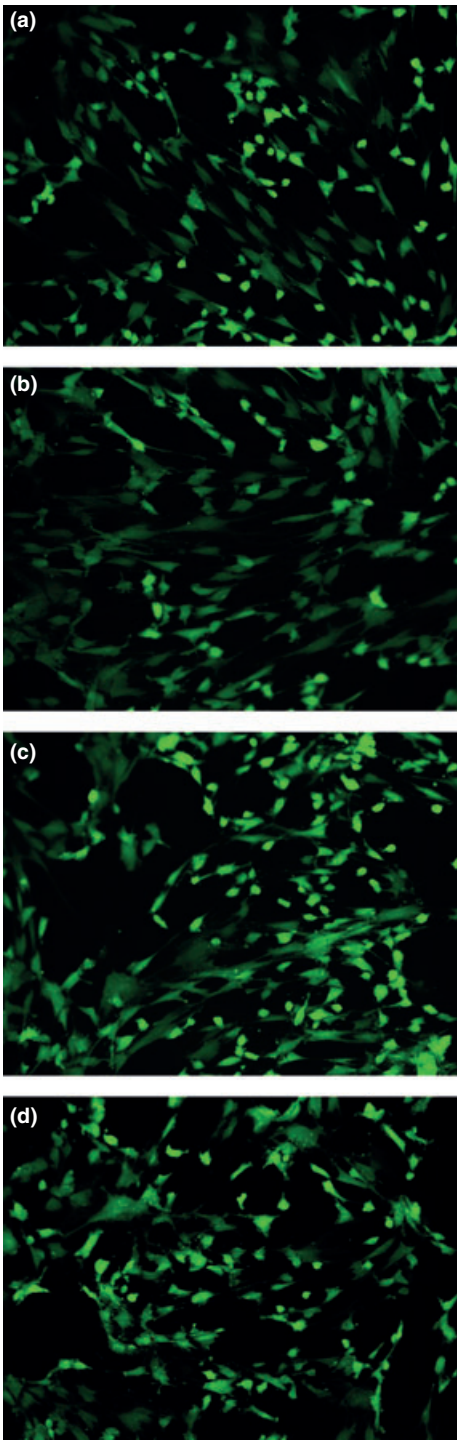

Fig. 3. Fluorescence microscopy images of human osteoblasts incubated with eluates of collagen (BioGide®) after staining with fluorescein diacetate (FDA) and propidium iodide (PI): note the green color due to staining with FDA, indicating living cells. The lack of a red color despite staining with PI indicates absence of dead cells. (a) 10 min; (b) 1 h; (c) 24 h and (d) control.

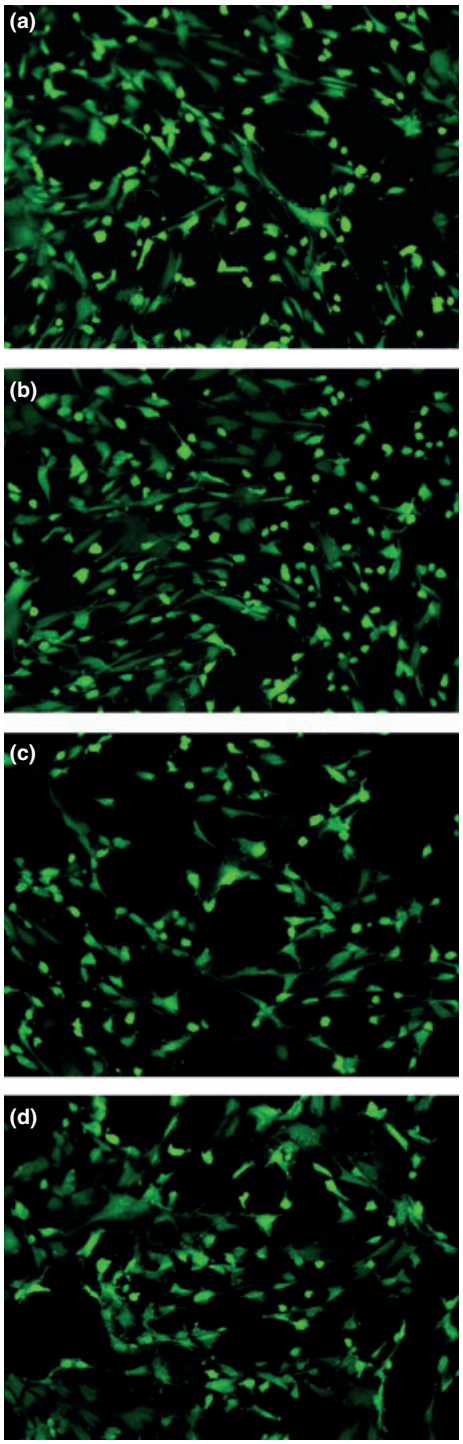

Fig. 4. Fluorescence microscopy images of human osteoblasts incubated with eluates of platelet rich fibrin (PRF) after staining with fluorescein diacetate (FDA) and propidium iodide (PI): note the green color due to staining with FDA, indicating living cells. The lack of a red color despite staining with PI indicates absence of dead cells. (a) 10 min; (b) 1 h; (c) 24 h and (d) control.

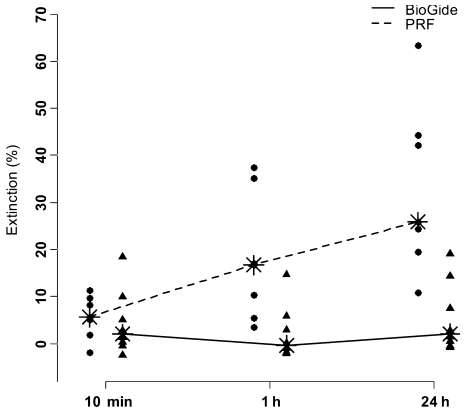

Fig. 5. Results from relative extinction with median values for lactate dehydrogenase assay for BioGide® and platelet-rich-fibrin (PRF) membranes after 10 min, 1, and 24 h.

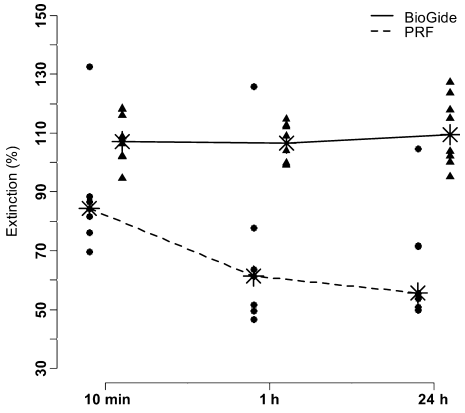

Fig. 6. Results from relative extinction with median values for MTT ([3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide]) assay for BioGide® and platelet-rich-fibrin (PRF) membranes after 10 min, 1, and 24 h.

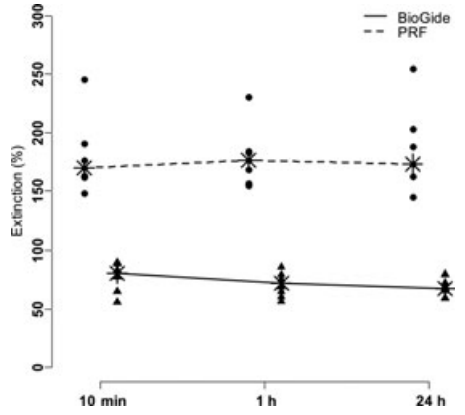

Fig. 7. Results from relative extinction with median values for water soluble tetrazolium (WST-1) (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay for BioGide® and platelet-rich-fibrin (PRF) membranes after 10 min, 1, and 24 h.

blasts on biomaterials (Anselme 2000). In living tissue the ECM offers a wide range of proteins which are able to bind to cell adhesion molecules and thus mediate the biochemical signal transduction from ECM to cytoskeleton (Juliano & Haskill 1993). *In vitro* experiments revealed that human osteoblast cells adhere preferentially to fibronectin more than to any other ECM protein. In particular, the interaction of $\alpha_5\beta_1$ integrin with specific amino acid sequences of fibronectin, such as arginine–glycine–aspartic acid (RGD) have been shown to be essential for

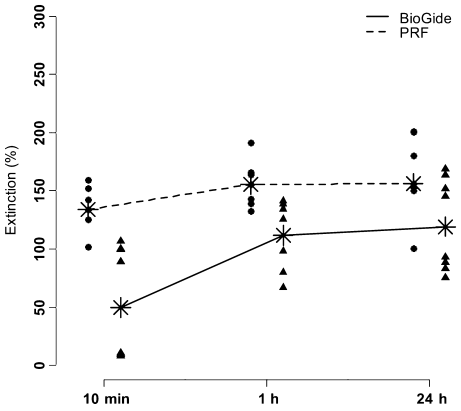

Fig. 8. Results from relative extinction with median values for BrdU (5-bromo-2-deoxyuridine) assay for BioGide® and platelet-rich-fibrin (PRF) membranes after 10 min, 1, and 24 h.

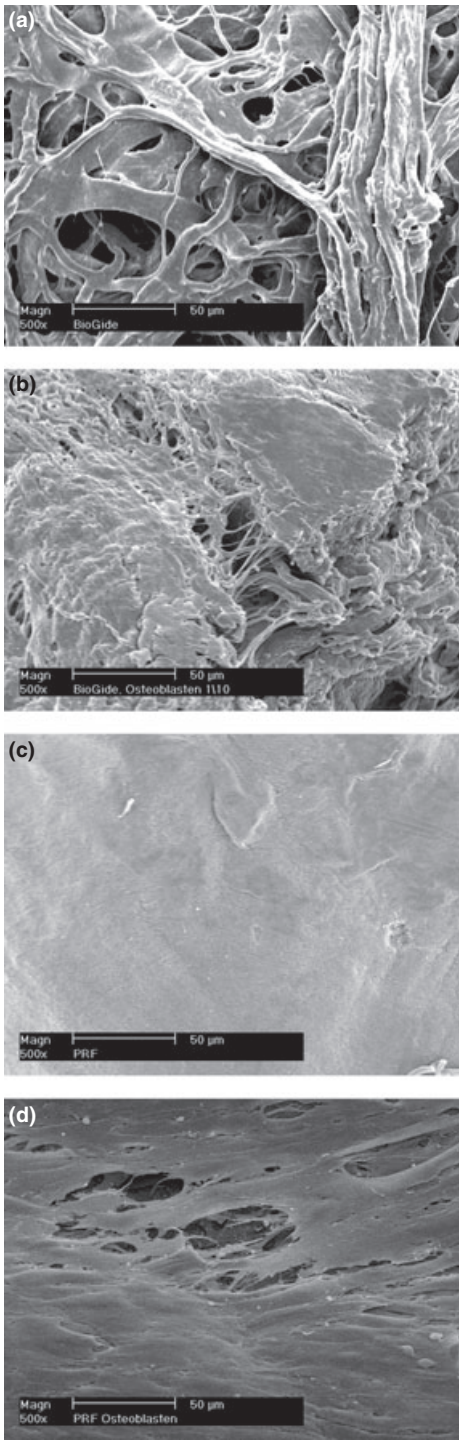

Fig. 9. Close-up scanning electron microscopic images of membranes seeded with human osteoblast cells and controls 7 days after seeding. The membranes were almost completely covered by cells, with an elongated morphology and numerous cell pseudopodia, suggesting good biocompatibility (a) collagen (BioGide®) without cells; (b) collagen (BioGide®) control with cells; (c) platelet rich fibrin (PRF) without cells; (d) PRF control with cells (scale bar = 50 µm).

Table 3. **Median, 25th and 75th percentile of differences in alkaline phosphatase activity (U/l) between day 1 and 36 of osteoblast cultures seeded on platelet rich fibrin (PRF) membranes (*n* = 10), PRF clots (*n* = 10), and BioGide® membranes (*n* = 10), respectively**

Membrane type	25th Percentile	50th Percentile	75th Percentile
PRF membrane	7.781	10.990	12.542
PRF clot	9.767	10.491	12.866
BioGide® membrane	0.705	1.202	1.950

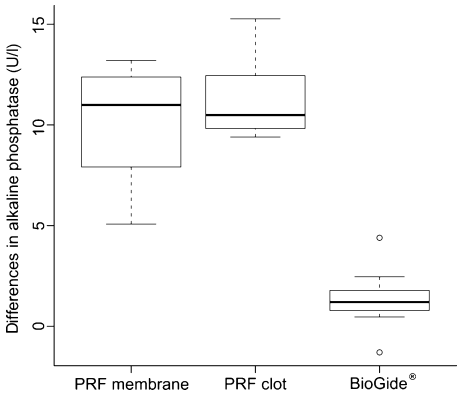

Fig. 10. Box plots showing the median, the upper, and the lower quartile of the differences in alkaline phosphatase activity (U/l) between day 1 and day 36 of osteoblasts seeded on platelet-rich-fibrin (PRF) membranes, PRF clots, and BioGide® membranes, respectively. Whiskers represent limits of normal variation while extreme values are marked as circles.

osteoblast cell adhesion, proliferation, and survival (Gronthos et al. 1997). But even morphogenesis and differentiation are regulated by the interaction of fibronectin and $\alpha_5\beta_1$ integrins of osteoblast cells (Moursi et al. 1997). In consequence, the use of specific antifibronectin antibodies to disturb the interaction leads to massive apoptosis and thus a survival signal via adhesion receptors seems to be likely (Globus et al. 1998).

It was known that the adhesive glycoprotein fibronectin is present in two forms; a soluble form in plasma and in an insoluble form in the ECM of many tissues. Hence, the occurrence of plasma fibronectin in blood derivates like PRF seems to be obvious. Recently it was found that nearly half of tissue fibronectin is of plasma origin and this finding suggests the importance of plasma as source for tissue fibronectin and in consequence for the modulation of cellular activities (Moretti et al. 2007). But it should be noted that fibronectin was even synthesized by different cell types *in vivo* and by bone cells *in vitro* (Steele et al. 1993).

The above-mentioned findings directly reflect the importance of adhesion for osteoblast cell function and thus may partially explain the higher cell proliferation of osteoblast cells on PRF than on collagen membranes in the present study (see Fig. 7).

A further possible reason for differing results concerning osteoblast cell proliferation may be caused by the lack of bone-specific proteins and a more physical than biological structure in BioGide®. Brightman et al. (2000) stated that the major disadvantage of most natural materials is the frequent loss of bioactive molecules by purification process as in BioGide®. In contrast PRF offers a more vital environment with ECM proteins which may help cells to adhere and to regulate cell function.

The ability of PRF to polymerize and form three-dimensional supramolecular assemblies with entrapped platelet cytokines (intrinsic cytokines) seem to be a fundamental advantage for tissue engineering (Dohan et al. 2006b). It could be shown that these cytokines have mitogenic properties for osteoblastic cells (Slater et al. 1995) and mediate the chemotaxis of undifferentiated multipotent MSCs and as a result lead to differentiation of cells to the osteoblastic phenotype (Gould et al. 2000). It has been shown that TGF-β in particular stimulates the synthesis of collagen and fibronectin in a variety of cell lines (Ignotz & Massague 1986) and furthermore, it has been found that TGF-β increases the expression of integrins that bind collagen, fibronectin, and vitronectin (Ignotz et al. 1989). In this context it was shown that fibrin delivery of cytokines like basic fibroblast growth factor and osteoblasts lead to higher bone formation than delivery of fibrin-containing osteoblasts alone (Park et al. 2006) and thus indicate the importance of growth factors for new bone formation. Recently it could be shown that PRF released autologous growth factors gradually and expressed strong and durable effects on prolif-

eration and differentiation of rat osteoblasts *in vitro* (He et al. 2009). In the present study these findings could be confirmed by examination of human osteoblast cultures on PRF clots, PRF membranes, and BioGide® membranes, respectively. It could be shown that the ALP, as an indicator of functional activity, was significantly higher in case of PRF clots and PRF membranes than in case of BioGide® membranes (see Fig. 10). ALP as an osteoblast-associated enzyme plays a particular role in the process of bone matrix calcification. Hence, the activity of ALP could be considered as a marker of osteogenic potential of osteoblasts (Halvorsen et al. 2001).

Thus, the abundance of cytokines present in PRF seems to function as a *drug delivery system* which has profound effects on cell development and composition of ECM. These facts could at least partially explain the better results of PRF membranes as shown in Figs 7 and 8. In case of BioGide®, a processed collagen membrane composed of porcine collagen type I and III, there is no vital microenvironment. Despite the fact that collagen fibers represent the major component in extracellular bone matrix which mediates cell-matrix attachment of osteogenic cells, the lack of bone-specific proteins leads to a purely physical scaffold nature of BioGide® and there is only a slight assumption that they may trap osteoinductive factors from bone (Koda & Bernfield 1984). Thus the ECM with entrapped cytokines in case of PRF may be of fundamental profit for tissue engineering. Conversely, the preparation of PRF leads to an entrapment of erythrocytes, which may explain the higher LDH values as shown in Fig. 5. Therefore, it should not be assumed from the LDH test that eluate from PRF membranes is more toxic to osteoblasts.

Tissue engineering of human osteoblasts is often difficult due to critical culturing conditions *in vitro*. In this context some essential considerations concerning the role of fibrin have been proposed by van Griensven et al. who found higher cell amounts of cultured osteoblast cells at an earlier time point than in "nonadhesive" cultures, possibly caused by the more adhesive property of outgrowing cells to the bottom of the culture dish mediated by fibrin glue. In contrast, when culturing without fibrin glue, osteoblasts' adhesive bonds are loosened due to movements during medium exchange (van Griensven et al. 2002).

Besides the interaction of bone cells and ECM structures, vascularization is of fundamental importance for bone tissue engineering although only a few remarks will be presented herein. It was known that the integrity of bone is strongly dependent on the interaction of bone cells and endothelium (Villars et al. 2000). In later stages of the wound healing process endothelial cells reorientate, invade, loosening the ECM, and then undergo tube formation (Folkman 1986). Fibrin was shown to influence the outgrowth of human microvascular endothelial cells, and it could be shown that the modulation of the fibrin structure markedly affects the extent and stability of capillary tube formation *in vitro* (Van Hinsbergh et al. 2001). The fibrin structure is largely influenced by polymerization conditions and thus has a significant impact in angiogenesis in course of the wound healing process. The progressive polymerization during centrifugation of PRF as described by Dohan et al. (2006b) might be a fundamental advantage of PRF scaffolds for *in vivo* bone tissue engineering applications.

Conclusion

The study objective was to compare two different ECM membrane systems by assessment of eluates for cultivating human osteoblast cells as well as by cultivating osteoblasts directly on membranes. Therefore, the analysis methods used were mainly intended to identify the vitality and proliferation of seeded cells. Herein, for the first time, it could be shown that PRF eluates support human osteoblast cell proliferation *in vitro*. Furthermore, it was shown that PRF is a suitable scaffold for cultivating human osteoblasts *in vitro*. Nevertheless both systems, PRF and collagen (BioGide®), seem to be suitable as scaffold materials for seeding by osteoblast cells *in vitro*.

Acknowledgements: The authors thank G. Otto for excellent technical assistance. This study was financed internally by the University of Schleswig-Holstein.

Author disclosure statement: The authors have declared no conflicts of interest.

References

Ahmed, T.A., Dare, E.V. & Hincke, M. (2008) Fibrin: a versatile scaffold for tissue engineering applications. *Tissue Engineering, Part B: Review* **14**: 199–215.

Anselme, K. (2000) Osteoblast adhesion on biomaterials. *Biomaterials* **21**: 667–681.

Anselme, K., Bigerelle, M., Noel, B., Dufresne, E., Judas, D., Iost, A. & Hardouin, P. (2000) Qualitative and quantitative study of human osteoblast adhesion on materials with various surface roughnesses. *Journal of Biomedical Materials Research* **49**: 155–166.

Bensaid, W., Triffitt, J.T., Blanchat, C., Oudina, K., Sedel, L. & Petite, H. (2003) A biodegradable fibrin scaffold for mesenchymal stem cell transplantation. *Biomaterials* **24**: 2497–2502.

Brightman, A.O., Rajwa, B.P., Sturgis, J.E., McCallister, M.E., Robinson, J.P. & Voytik-Harbin, S.L. (2000) Time-lapse confocal reflection microscopy of collagen fibrillogenesis and extracellular matrix assembly *in vitro*. *Biopolymers* **54**: 222–234.

Brunner, E., Domhof, S. & Langer, F. (2002) Nonparametric Analysis of Longitudinal Data in Factorial Experiments. New York: Wiley.

Burchardt, H. (1983) The biology of bone graft repair. *Clinical Orthopaedics and Related Research* **174**: 28–42.

Catelas, I., Sese, N., Wu, B.M., Dunn, J.C., Helgerson, S. & Tawil, B. (2006) Human mesenchymal stem cell proliferation and osteogenic differentiation in fibrin gels *in vitro*. *Tissue Engineering* **12**: 2385–2396.

Choukroun, J., Adda, F., Schoeffler, C. & Vervelle, A. (2001) An opportunity in perio-implantology: the PRF (in French). *Implantodontie* **42**: 55–62.

Damsky, C.H. (1999) Extracellular matrix-integrin interactions in osteoblast function and tissue remodeling. *Bone* **25**: 95–96.

Dohan, D.M., Choukroun, J., Diss, A., Dohan, S.L., Dohan, A.J., Mouhyi, J. & Gogly, B. (2006a) Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part I: technological concepts and evolution. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology* **101**: e37–e44.

Dohan, D.M., Choukroun, J., Diss, A., Dohan, S.L., Dohan, A.J., Mouhyi, J. & Gogly, B. (2006b) Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part II: platelet-related biologic features. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology* **101**: e45–e50.

Folkman, J. (1986) How is blood vessel growth regulated in normal and neoplastic tissue? G.H.A. Clowes memorial Award lecture. *Cancer Research* **46**: 467–473.

Frohlich, M., Grayson, W.L., Wan, L.Q., Marolt, D., Drobnic, M. & Vunjak-Novakovic, G. (2008) Tissue engineered bone grafts: biological requirements, tissue culture and clinical relevance. *Current Stem Cell Research & Therapy* **3**: 254–264.

Gassling, V., Douglas, T., Warnke, P.H., Acil, Y., Wiltfang, J. & Becker, S.T. (2010) Platelet-rich fibrin membranes as scaffolds for periosteal tissue engineering. *Clinical Oral Implants Research* **21**: 543–549.

Globus, R.K., Doty, S.B., Lull, J.C., Holmuhamedov, E., Humphries, M.J. & Damsky, C.H. (1998) Fibronectin is a survival factor for differentiated osteoblasts. *Journal of Cell Science* **111** (Part 10): 1385–1393.

Gould, S.E., Rhee, J.M., Tay, B.K.B., Otsuka, N.Y. & Bradford, D.S. (2000) Cellular contribution of bone graft to fusion. *Journal of Orthopaedic Research* **18**: 920–927.

van Griensven, M., Zeichen, J., Tschernig, T., Seekamp, A. & Pape, H.C. (2002) A modified method to culture human osteoblasts from bone tissue specimens using fibrin glue. *Experimental and Toxicologic Pathology* **54**: 25–29.

Gronthos, S., Stewart, K., Graves, S.E., Hay, S. & Simmons, P.J. (1997) Integrin expression and function on human osteoblast-like cells. *Journal of Bone and Mineral Research* **12**: 1189–1197.

Gruber, R., Karreth, F., Frommlet, F., Fischer, M.B. & Watzek, G. (2003) Platelets are mitogenic for periosteum-derived cells. *Journal of Orthopaedic Research* **21**: 941–948.

Halvorsen, Y.D., Franklin, D., Bond, A.L., Hitt, D. C., Auchter, C., Boskey, A.L., Paschalis, E.P., Wilkison, W.O. & Gimble, J.M. (2001) Extracellular matrix mineralization and osteoblast gene expression by human adipose tissue-derived stromal cells. *Tissue Engineering* **7**: 729–741.

He, L., Lin, Y., Hu, X., Zhang, Y. & Wu, H. (2009) A comparative study of platelet-rich fibrin (PRF) and platelet-rich plasma (PRP) on the effect of proliferation and differentiation of rat osteoblasts *in vitro*. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology* **108**: 707–713.

Ignotz, R.A., Heino, J. & Massague, J. (1989) Regulation of cell adhesion receptors by transforming growth factor-beta. Regulation of vitronectin receptor and LFA-1. *The Journal of Biological Chemistry* **264**: 389–392.

Ignotz, R.A. & Massague, J. (1986) Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *The Journal of Biological Chemistry* **261**: 4337–4345.

Isogai, N., Landis, W.J., Mori, R., Gotoh, Y., Gerstenfeld, L.C., Upton, J. & Vacanti, J.P. (2000) Experimental use of fibrin glue to induce site-directed osteogenesis from cultured periosteal cells. *Plastic and Reconstructive Surgery* **105**: 953–963.

Juliano, R.L. & Haskill, S. (1993) Signal transduction from the extracellular matrix. *The Journal of Cell Biology* **120**: 577–585.

Kasperk, C., Wergedal, J., Strong, D., Farley, J., Wangerin, K., Gropp, H., Ziegler, R. & Baylink, D.J. (1995) Human bone cell phenotypes differ depending on their skeletal site of origin. *Journal of Clinical Endocrinology & Metabolism* **80**: 2511–2517.

Koda, J.E. & Bernfield, M. (1984) Heparan sulfate proteoglycans from mouse mammary epithelial cells. Basal extracellular proteoglycan binds specifically to native type I collagen fibrils. *The Journal of Biological Chemistry* **259**: 11763–11770.

Kunzler, T.P., Drobek, T., Schuler, M. & Spencer, N.D. (2007) Systematic study of osteoblast and fibroblast response to roughness by means of surface-morphology gradients. *Biomaterials* **28**: 2175–2182.

Lind, M. (1998) Growth factor stimulation of bone healing. Effects on osteoblasts, osteomies, and implants fixation. *Acta Orthopaedica Scandinavica. Supplementum* **283**: 2–37.

Lohmann, C.H., Bonewald, L.F., Sisk, M.A., Sylvia, V.L., Cochran, D.L., Dean, D.D., Boyan, B.D. & Schwartz, Z. (2000) Maturation state determines the response of osteogenic cells to surface roughness and 1,25-dihydroxyvitamin D3. *Journal of Bone and Mineral Research* **15**: 1169–1180.

Moretti, F.A., Chauhan, A.K., Iaconcig, A., Porro, F., Baralle, F.E. & Muro, A.F. (2007) A major fraction of fibronectin present in the extracellular matrix of tissues is plasma-derived. *The Journal of Biological Chemistry* **282**: 28057–28062.

Mosesson, M.W. (2005) Fibrinogen and fibrin structure and functions. *Journal of Thrombosis and Haemostasis* **3**: 1894–1904.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* **65**: 55–63.

Moursi, A.M., Globus, R.K. & Damsky, C.H. (1997) Interactions between integrin receptors and fibronectin are required for calvarial osteoblast differentiation *in vitro*. *Journal of Cell Science* **110** (Part 18): 2187–2196.

Naji, A. & Harmand, M.F. (1990) Study of the effect of the surface state on the cytocompatibility of a Co-Cr alloy using human osteoblasts and fibroblasts. *Journal of Biomedical Materials Research* **24**: 861–871.

Park, M.S., Kim, S.S., Cho, S.W., Choi, C.Y. & Kim, B.S. (2006) Enhancement of the osteogenic efficacy of osteoblast transplantation by the sustained delivery of basic fibroblast growth factor. *Journal of Biomedical Materials Research, Part B: Applied Biomaterials* **79**: 353–359.

Santos, M.I. & Reis, R.L. (2010) Vascularization in bone tissue engineering: physiology, current strategies, major hurdles and future challenges. *Macromolecular Bioscience* **10**: 12–27.

Schliephake, H. (2002) Bone growth factors in maxillofacial skeletal reconstruction. *The International Journal of Oral and Maxillofacial Surgery* **31**: 469–484.

Slater, M., Patava, J., Kingham, K. & Mason, R.S. (1995) Involvement of platelets in stimulating osteogenic activity. *Journal of Orthopaedic Research* **13**: 655–663.

Steele, J.G., McFarland, C., Dalton, B.A., Johnson, G., Evans, M.D., Howlett, C.R. & Underwood, P. A. (1993) Attachment of human bone cells to tissue culture polystyrene and to unmodified polystyrene: the effect of surface chemistry upon initial cell attachment. *Journal of Biomaterials Science, Polymer Edition* **5**: 245–257.

Trombi, L., D'Alessandro, D., Pacini, S., Fiorentino, B., Scarpellini, M., Fazzi, R., Galimberti, S., Guazzini, S. & Petrini, M. (2008) Good manufacturing practice-grade fibrin gel is useful as a scaffold for human mesenchymal stromal cells and supports *in vitro* osteogenic differentiation. *Transfusion* **48**: 2246–2251.

Vacanti, C.A. & Vacanti, J.P. (2000) The science of tissue engineering. *Orthopedic Clinics of North America* **31**: 351–356.

Van Hinsbergh, V.W., Collen, A. & Koolwijk, P. (2001) Role of fibrin matrix in angiogenesis. *Annals of the New York Academy of Sciences* **936**: 426–437.

Villars, F., Bordenave, L., Bareille, R. & Amedee, J. (2000) Effect of human endothelial cells on human bone marrow stromal cell phenotype: role of VEGF? *Journal of Cellular Biochemistry* **79**: 672–685.

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.

Zipfel, G.J., Guiot, B.H. & Fessler, R.G. (2003) Bone grafting. *Neurosurgical Focus* **14**: e8.