Tissue engineering of composite grafts: Cocultivation of human oral keratinocytes and human osteoblast-like cells on laminin-coated polycarbonate membranes and equine collagen membranes under different culture conditions

R. Glaum, M. Wiedmann-Al-Ahmad, U. Huebner, R. Schmelzeisen
Department of Oral and Maxillofacial Surgery, Albert Ludwigs University, Freiburg, Germany

Received 21 April 2008; revised 25 February 2009; accepted 19 May 2009
Published online 16 July 2009 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.32575

Abstract: In complex craniomaxillofacial defects, the simultaneous reconstruction of hard and soft tissue is often necessary. Until now, oral keratinocytes and osteoblast-like cells have not been cocultivated on the same carrier. For the first time, the cocultivation of human oral keratinocytes and human osteoblast-like cells has been investigated in this study. Different carriers (laminin-coated polycarbonate and equine collagen membranes) and various culture conditions were examined. Human oral keratinocytes and human osteoblast-like cells from five patients were isolated from tissue samples, seeded on the opposite sides of the carriers and cultivated for 1 and 2 weeks under static conditions in an incubator and in a perfusion chamber. Proliferation and morphology of the cells were analyzed by EZ4U-tests, light microscopy, and scanning electron microscopy. Cocultivation of both cell-types seeded on one carrier was possible. Quantitative and qualitative growth was significantly better on collagen membranes when compared with laminin-coated polycarbonate membranes independent of the culture conditions. Using perfusion culture in comparison to static culture, the increase of cell proliferation after 2 weeks of cultivation when compared with the proliferation after 1 week was significantly lower, independent of the carriers used. In conclusion, the contemporaneous cultivation of human oral keratinocytes and human osteoblast-like cells on the same carrier is possible, a prerequisite for planned *in vivo* studies. As carrier collagen is superior to laminin-coated polycarbonate membranes. Regarding the development over time, the increase of proliferation rate is lower in perfusion culture. Examinations of cellular differentiation over time under various culture conditions will be subject of further investigations. © 2009 Wiley Periodicals, Inc. J Biomed Mater Res 93A: 704–715, 2010

Key words: cocultivation; oral keratinocytes; osteoblasts; biomaterials; tissue engineering

INTRODUCTION

Many studies reported the examination and optimization of tissue engineering procedures to replace single tissues. These methods gain in importance especially when other donor sites are rare or bearing the risk of severe donor site morbidity, transmission of disease, or problems in immuncompatibility.[1,2]

Grafts for, for example, head and neck reconstructions often require hard and soft tissue. Apart from the hard tissue component which is addressed by different approaches in many cases an intraoral lining by oral mucosa needs to be achieved.[3–5] Lauer et al. (2001) introduced a radial forearm flap prelaminated with *in vitro* cultured oral keratinocytes.[6] Besides the mucosa-like lining in the oral cavity, a primary wound closure could be achieved at the donor site. Schultze-Mosgau et al. (2004) transplanted preconfluent oral keratinocytes on the gracilis muscle of Wistar rats and achieved a uniform multilayered oral epithelium coverage.[7]

For the ideal transplant in the field of combined hard and soft tissue reconstruction by the means of tissue engineering, different cell types in coculture are necessary. Up to now, there are only few approaches of combined hard and soft tissue substitutes. Neovius and Kratz (2003) described the cocultivation of dermal keratinocytes and elastic chondrocytes for ear reconstruction.[8] Many attempts were performed to create a dermal and an epidermal layer to treat full-thickness defects of the skin. For this purpose, dermal keratinocytes and with fibroblasts-seeded dermal equivalents were combined.[9–11] To the best of our knowledge until now, oral keratinocytes and osteoblast-like cells have not been investigated on the same carrier even if they

Correspondence to: M. Wiedmann-Al-Ahmad; e-mail: wiedmannalahmad@t-online.de

are common cells in the head and neck region, whereas the combination of oral keratinocytes and fibroblasts has been already examined.[12,13]

The aim of this study was to investigate the cocultivation of human oral keratinocytes and human osteoblast-like cells on laminin-coated polycarbonate membranes and equine collagen membranes. Polycarbonate membranes and collagen are described to support the growth of human oral keratinocytes and human osteoblast-like cells.[13–16] The laminin-coating of polycarbonate membranes seems to enhance the growth of human osteoblast-like cells and also the attachment of oral keratinocytes to culture dishes.[17] Furthermore, we compared the influence of two different culture conditions (static culture in an incubator vs. perfusion culture system) on the growth of the cocultivated cells.

The perfusion culture system guarantees the continuous exchange of medium, constant removal of metabolic waste and has been used for the cultivation of a variety of cells and tissues.[18–21] These circumstances have a positive influence on growth of osteoblast-like cells as well as on oral keratinocytes.[18,22–24] Especially, less dedifferentiation of the cells as demonstrated by Strehl et al. (2005) for the long-term cultivation of cartilage, multilayered growth, and deposition of extracellular matrix of osteoblast-like cells were described.[18,25,26] The latter is meant to be due to fluid-flow derived shear stress.[27–29] The first successful *in vitro* cocultivation of human oral keratinocytes and human osteoblast-like cells on the same scaffold would be of main interest in the field of tissue engineering. It is a necessary prerequisite for planned *in vivo* studies and a further step to optimize craniomaxillofacial defect reconstructions.

MATERIALS AND METHODS

Isolation and cultivation of cells

After informed consent (Ethic commission of the Medical Faculty of Freiburg University, Germany) samples of bone and oral mucosa were harvested from five patients during oral and maxillofacial interventions and placed into sterile 0.9% NaCl solution.

For the cultivation of osteoblast-like cells, samples of bone were minced into pieces of 1–2 mm diameter and placed into culture flasks (25 cm^3, Greiner, Frickenhausen, Germany) using Opti-MEM1 + Glutamax (Opti Minimal Essential Medium, Gibco Invitrogen Life Technologies, Paisley, UK) supplemented with 10% foetal calf serum (FCS, PAA Laboratories, Linz, Austria), 2% 1*M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES, Roth, Karlsruhe, Germany), 1000 mg/L $CaCl_2$ (Roth, Karlsruhe, Germany), and 1% penicillin/streptomycin solution (PAA Laboratories, Linz, Austria). The explants were incubated at 37°C in a humidified atmosphere with 5% CO_2 (Heraeus Instruments, Osterode, Germany). After reaching confluence cells were trypsinized (0.5% trypsine in PBS, PAA Laboratories, Linz, Austria) and transferred into 75 cm^3 culture flasks (Greiner, Frickenhausen, Germany). RPMI 1640 (Gibco Laboratories Life Technologies, NY) containing 10% FCS, 1% penicillin/streptomycin solution, and 5% HEPES was used as medium for further cultivation. Cells were used for experiments after second passage.

For the cultivation of oral keratinocytes, samples of mucosa were minced into pieces of 1–2 mm diameter and placed into culture flasks (75 cm^3, Greiner, Frickenhausen, Germany). The used medium consisted of 750 mL Dulbecco's Modified Eagle Medium (DMEM) with 1 g L-Glucose (Bio Whittaker, Cambrex Bio Science Verviers, Verviers, Belgium), 250 mL Nutrient Mixture Ham's F-12 with Glutamine (Gibco Invitrogen Life Technologies, Paisley, UK), 10% FCS, 2% HEPES, and 1% penicillin/streptomycin solution supplemented with 293 g/L L-glutamine (PAA Laboratories, Linz, Austria), 10 μg/L epidermal growth factor (Sigma, Roedermark, Germany), 5 mg/L insulin (Sigma, Roedermark, Germany), 5 mg/L human transferrin (Sigma, Roedermark, Germany), 1.36 μg/L triiodotyronine (Sigma, Roedermark, Germany), 0.2 mg/L hydrocortisone (Sigma, Roedermark, Germany), 0.0085 mg/L cholera-toxine (Sigma, Roedermark, Germany), 0.024 g/L adenine (Sigma, Roedermark, Germany), and 10.5 mg/L spermine (Sigma, Roedermark, Germany). The explants were incubated at 37°C in humidified atmosphere with 5% CO_2 (Heraeus Instruments, Osterode, Germany). Cells were used for experiments after the first passage.

Cells were trypsinized and seeded on different carriers. Cells seeded in culture dishes served as controls and for cell characterization analysis.

Carriers

Polycarbonate membranes (diameter 13 mm) with a pore size of 0.4 μm (Millipore, Minucells, and Minutissue, Bad Abbach, Germany) and equine collagen membranes (Tissue Foil E®, Baxter, Resorba, Nuremberg, Germany) were used. The polycarbonate membranes were coated with laminin (Sigma-Aldrich, Deisenhofen, Germany). The coating was performed with a concentration of 1–2 $\mu g/cm^2$ on both sides.

Cell seeding

50 μL osteoblast-like cells (2×10^6 cells/mL) were seeded onto the membranes fixed in carrier-devices (Minusheet, Minucells and Minutissue, Bad Abbach, Germany) and incubated for 24 h at 37°C/5% CO_2 atmosphere, followed by seeding of oral keratinocytes in the same way on the opposite side of the membrane. After further incubation for 24 h at 37°C/5% CO_2 atmosphere half of the carriers were placed into the perfusion chamber (Minucells and Minutissue, Bad Abbach, Germany; Fig. 1). The medium is transported by a peristaltic pump (Ismatec, Glattbrugg-Zurigo, Switzerland) with a flow rate of 3 mL/h

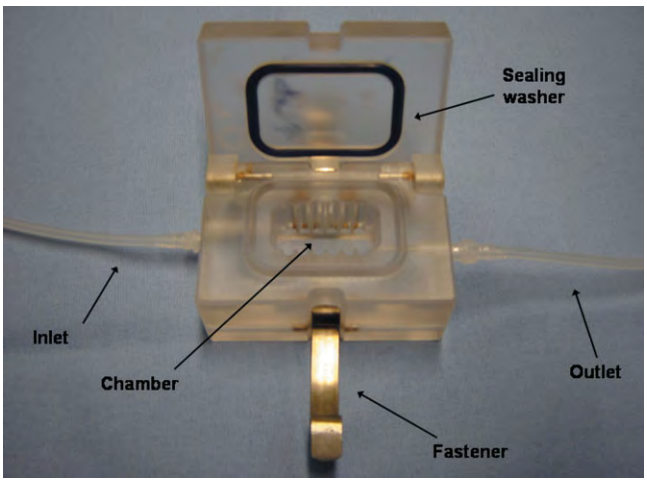

Figure 1. Perfusion chamber (Minucells and Minutissue, Bad Abbach, Germany) with inlet and outlet tube. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

from the bottle with fresh medium to the culture container which is placed on a heating plate (37°C). The waste medium is collected in another bottle (Fig. 1). The other carriers were incubated at 37°C/5% CO_2 atmosphere under static conditions. Additionally, for cell characterization plates were seeded with 1×10^5 cells and incubated for 1 week at 37°C in 5% CO_2 atmosphere. In static culture, RPMI 1640 containing 10% FCS, 1% penicillin/streptomycin solution, and 2% HEPES was used. For perfusion culture experiments, 4% HEPES instead of 2% was used to avoid an increase of pH outside the incubator. After 7 and 14 days, samples were examined by cell proliferation analysis, light microscopy, and scanning electron microscopy.

Cell proliferation analysis

The cell proliferation was analyzed by the nonradioactive assay EZ4U (Easy for you, Biomedica, Wien, Austria). This method is based on the finding that living cells are capable to reduce slightly yellow colored tetrazolium salts into intense red colored formazan derivatives by an intracellular reduction system, mostly located in the mitochondria. These formazan derivates are excreted into the culture medium and the absorbance can be measured with a microplate reader. The amount of colored formazan derivatives correlates with the amount of living cells in the sample. The proliferation assay was carried out according to the manufacturers' instructions.

Scanning electron microscopy

The cell colonization analysis was assessed by scanning electron microscopy. For scanning electron microscopy, the samples were fixed in 8% formaldehyde for 2 days followed by dehydration in graded alcohol (30, 50, 70, 80, 90, each one time and two times in 99.8%). After critical point drying (CPD 030, BAL-TEC, Wallruf, Germany), according to standard procedure using liquid carbon dioxide, the samples were sputtered (SCD 050, BAL-TEC, Wallruf, Germany) with goldpalladium (Provac, Oestrich-Winkel, Germany). The samples were examined via Zeiss Leo 32 scanning electron microscope (Zeiss, Kochern, Germany) at 10 KV. Images were digitalized.

Light microscopy

For light microscopy, samples were fixed in 8% formaldehyde. Over an amounting alcohol gradient, the segments were dehydrated and embedded in 2-hydroxyethyl-methacrylate (Technovit 8100, Haereus-Kulzer, Wehrheim, Germany). By microtome (Leica, Nussloch, Deutschland), thin sections of ~2.5 μm were manufactured and stained with Nucleus Fast Red 0.3%/5% $Al_2(SO_4)_3$ in Aqua dest. ($C_{14}H_8NNaO_7S$, Fluca Chemie GmbH, Buchs, Switzerland). The sections were examined by light microscopy (Axioskop, Zeiss, Oberkochen, Germany) with 10×, 20×, 50×, and 100× magnification. Images were digitalized.

Cell characterization

For cell characterization, cells were seeded in 24-well-plates and examined after a cultivation period of 7 days. Osteoblast-like cells were examined for alkaline phosphatase and osteocalcin, whereas oral keratinocytes for cytokeratine 13 and 19.

Assay for osteocalcin

Osteocalcin is exclusively synthesized by osteoblasts and is believed to prevent premature mineralization of newly formed, but yet disorganized bone matrix.[30] For the quantification of osteocalcin in the cell culture, supernatant of human osteoblast-like cells the METRA osteocalcin EIA Kit (Quidel Corporation, San Diego, CA) was performed according to the manufacturer's instructions. The absorbance was measured at 405 nm.

Alkaline phosphatase assay

For staining of osteoblast-like cells an alkaline phosphatase assay kit (Sigma, Roedermark, Germany) was used. The culture dishes were air dried, fixed in citrate-aceton-formaldehyde solution for 30 s, and rinsed gently with Aqua dest. Cells were incubated with alkaline phosphatase staining solution for 15 min protected from direct light and washed with Aqua dest. The citrate-aceton-formaldehyde solution as well as the alkaline phosphatase staining solution were prepared according to the manufacturer's instructions. The culture dishes were counterstained with neutralred for 5 min and rinsed with Aqua dest. Positive staining for alkaline phosphatase (red-violet) was identified by light microscopy and evaluated by morphometry (analySIS, Soft Imaging System GmbH, Münster, Germany).

TABLE I
Cell Proliferation Analysis of Human Oral Keratinocytes and Human Osteoblast-Like Cells After a Cultivation Period of 1 Week and 2 Weeks on Polycarbonate Membranes and on Collagen Membranes

	1 Week [OD 450 nm]	2 Weeks [OD 450 nm]
Polycarbonate		
Perfusion	0.15 ± 0.19	0.14 ± 0.09
Incubator	0.14 ± 0.19	0.37 ± 0.3
Collagen		
Perfusion	0.71 ± 0.12	0.74 ± 0.14
Incubator	0.61 ± 0.2	1.03 ± 0.46

The constructs were cultivated under static conditions (incubator) and in perfusion chamber (perfusion), respectively. Shown are mean and standard deviations of the measured optical densities.

Detection of cytokeratines 13 and 19

Cytokeratines 13 and 19 were examined immunhistochemically using the avidine-biotine-method. The culture dishes were washed in PBS for 5 min, fixed with 70% ethanol for 1 h, washed in PBS for 5 min, allowed to air-dry, and washed again in PBS. After an incubation with 0.3% H_2O_2 in methanol for 10 min, unspecific immune reactions were blocked with 10% bovine serum albumin (Merck, Darmstadt, Germany) for 10 min, before the murine antibody against human Cytokeratines 13 or 19 (Sigma, Roedermark, Germany) was added and incubated over night at 4°C. The detection of the attached antibody was performed using the ABC-Kit (Vectastain, Burlingame, CA) according to the manufacturer's instructions. Staining reaction was achieved by DAB-Chromogen (4 mg 3,3′-diaminobenzidintetrahydrochloride ($C_{12}H_{14}N_4 \cdots 4HCl$, DAB, Sigma, Roedermark, Germany) in 10 mL 0.05*M* Tris-(hydroxylmethyl)aminomethane ($C_4H_{11}NO_3$, Trisbuffer, Roth, Karlsruhe, Germany; pH 7.2–7.4 with 17 µL 30% H_2O_2), added for 15–25 min. Washing with PBS and counterstaining with hemalaun (Mayers Hämalaunlösung, Merck, Darmstadt, Germany) for 2 min followed. Afterwards the samples were washed with Aqua dest. and air-dried. Positive staining (brown cytoplasm and blue nuclei) was identified by light microscopy.

Statistics

The results of the proliferation test were analyzed by repeated measures ANOVA (SAS 8.2 PROC GLM) after converting the data according to $\log(0.3 + x) - \log(0.3)$ to optimize the skewness of the distribution. Furthermore, student's *t*-tests were performed.

RESULTS

Cell proliferation and vitality

The vitality and proliferation capacity of the cells from five different patients seeded onto different membranes and cultured under static and perfusion culture conditions were studied by the EZ4U – EASY FOR YOU test. The average of the measured absorbance (OD_{450nm}) after 1 and 2 weeks of cultivation is shown in Table I and Figure 2 in dependence on the two different membranes and the culture conditions.

Cells seeded on collagen membranes showed significantly higher proliferation rates when compared with cells seeded on laminin-coated polycarbonate membranes ($p < 0.001$). An overall difference between culture conditions is not detectable ($p = 0.4$).

On both investigated materials, a significant influence of culture conditions on the increase of cell viability between week 1 and week 2 could be observed ($p < 0.05$). Under perfusion culture conditions, cell vitality on polycarbonate and collagen membranes was stable between 1 and 2 weeks of cultivation ($p = 0.482$, $p = 0.448$, respectively), whereas under static conditions a significant increase could be detected ($p < 0.001$, $p = 0.001$, respectively).

Light microscopy

For light microscopy, cultivated membranes were stained with nucleus fast red. Nuclei appeared red, whereas cytoplasm and the collagen membrane were slight rose. The polycarbonate membrane is not stained. The different celltypes cannot be distinguished. Cells on both sides and multilayered cell growth were visible more frequently on collagen

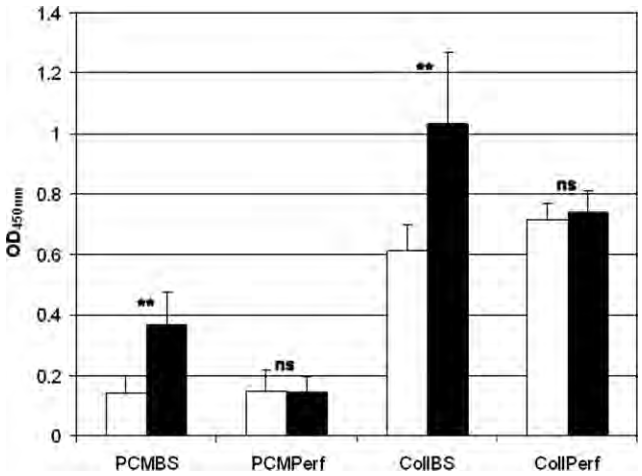

Figure 2. Cell proliferation analysis of human oral keratinocytes and human osteoblast-like cells after a cultivation period of 7 days (white) and 14 days (black) on polycarbonate membranes (PCM) and on collagen membranes (Coll). The constructs were cultivated under static conditions (BS) and in perfusion chamber (Perf), respectively. Significant differences were detectable between 7 days and 14 days of cultivation under static conditions (**). There were no significant differences in perfusion culture (ns). Error bars represent 95% confidence interval.

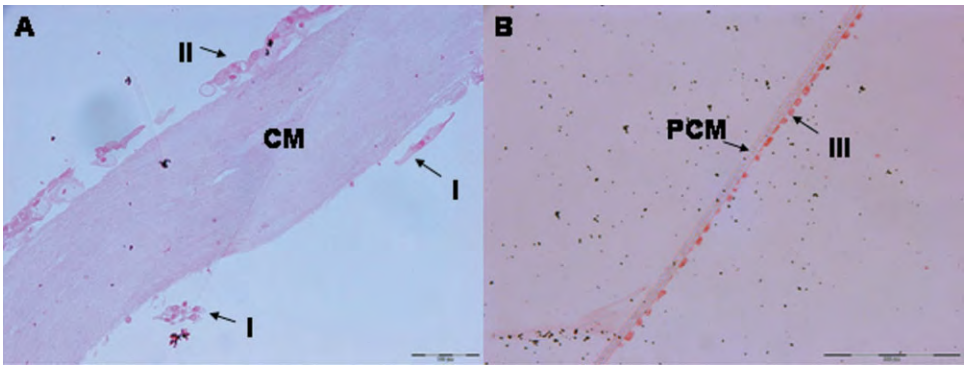

Figure 3. Light microscopy of collagen membrane (A) and laminin-coated polycarbonate membrane (B) seeded with human oral keratinocytes and human osteoblast-like cells after a cultivation period of 1 week in perfusion culture. Cells on one side (I) and a multilayer of cells on the other side (II) of the collagen membrane (Coll) are visible. On polycarbonate membrane (PCM) numerous cells (III) on one side are detectable. Magnification ×20. Nucleus fast red staining. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

membranes when compared with polycarbonate membranes (Fig. 3). Morphological differences between static and perfusion culture as well as between cultivation periods for 1 week and 2 weeks were not detectable by light microscopy (data not shown).

Scanning electron microscopy

The surface of the membranes with respect to cell morphology and growth pattern was examined by scanning electron microscopy. Although osteoblast-like cells appeared as longish cells arranged like a draught of fishes in confluent layers [Fig. 4(A)], oral keratinocytes appeared more compact, polygonal, and flat and showed a cobble stone pattern when confluent [Fig. 4(B)].

Cell growth was detectable on both sides of polycarbonate and collagen membranes after cultivation under static conditions as well as in perfusion culture (Fig. 5–8).

On polycarbonate membranes isolated keratinocytes [Fig. 5(A)] and osteoblasts [Fig. 5(B)] or small conglomerates of cells were visible on both sides after 1 week of cultivation in the incubator [Fig. 5(A,B)]. After 2 weeks, more conglomerates of oral keratinocytes were present [Fig. 5(C)], whereas the number of osteoblast-like cells did not increase [Fig. 5(D)].

The results after a cultivation period for 1 week in perfusion chamber were similar when compared with the cultivation under static conditions: isolated cells and some conglomerates on both sides of the membranes were visible [Fig. 6(A,B)]. Only isolated, partly detached keratinocytes could be observed after a cultivation period of 2 weeks in perfusion culture [Fig. 6(C)], whereas the number of osteoblast-like cells increased [Fig. 6(D)].

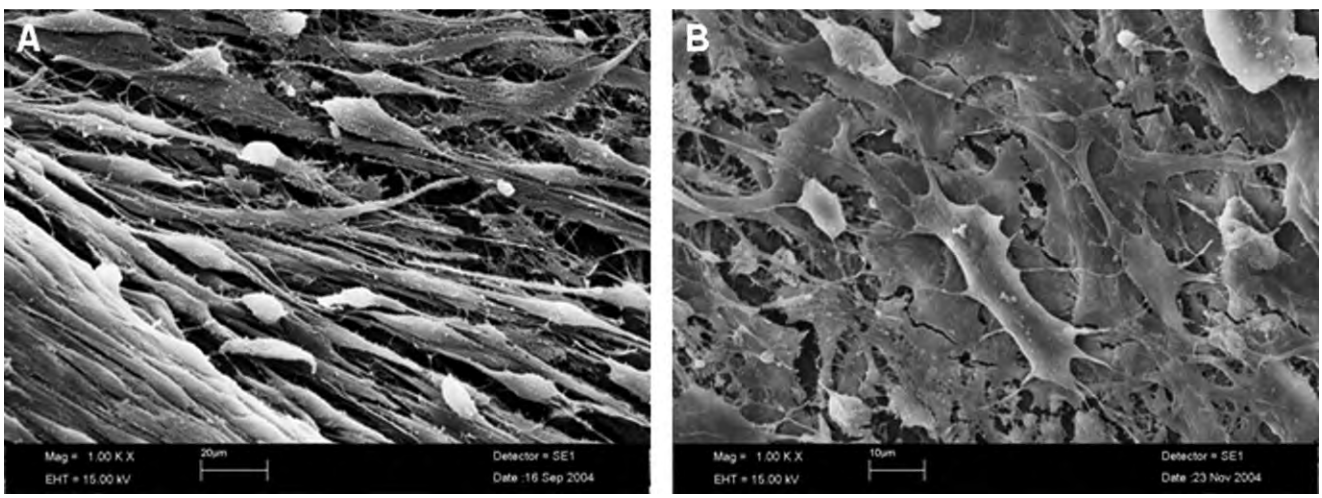

Figure 4. Scanning electrone micrographs of osteoblast-like cells on polycarbonate membrane (A) and of oral keratinocytes on collagen membrane (B) after 1 week under static conditions. Magnification ×1000.

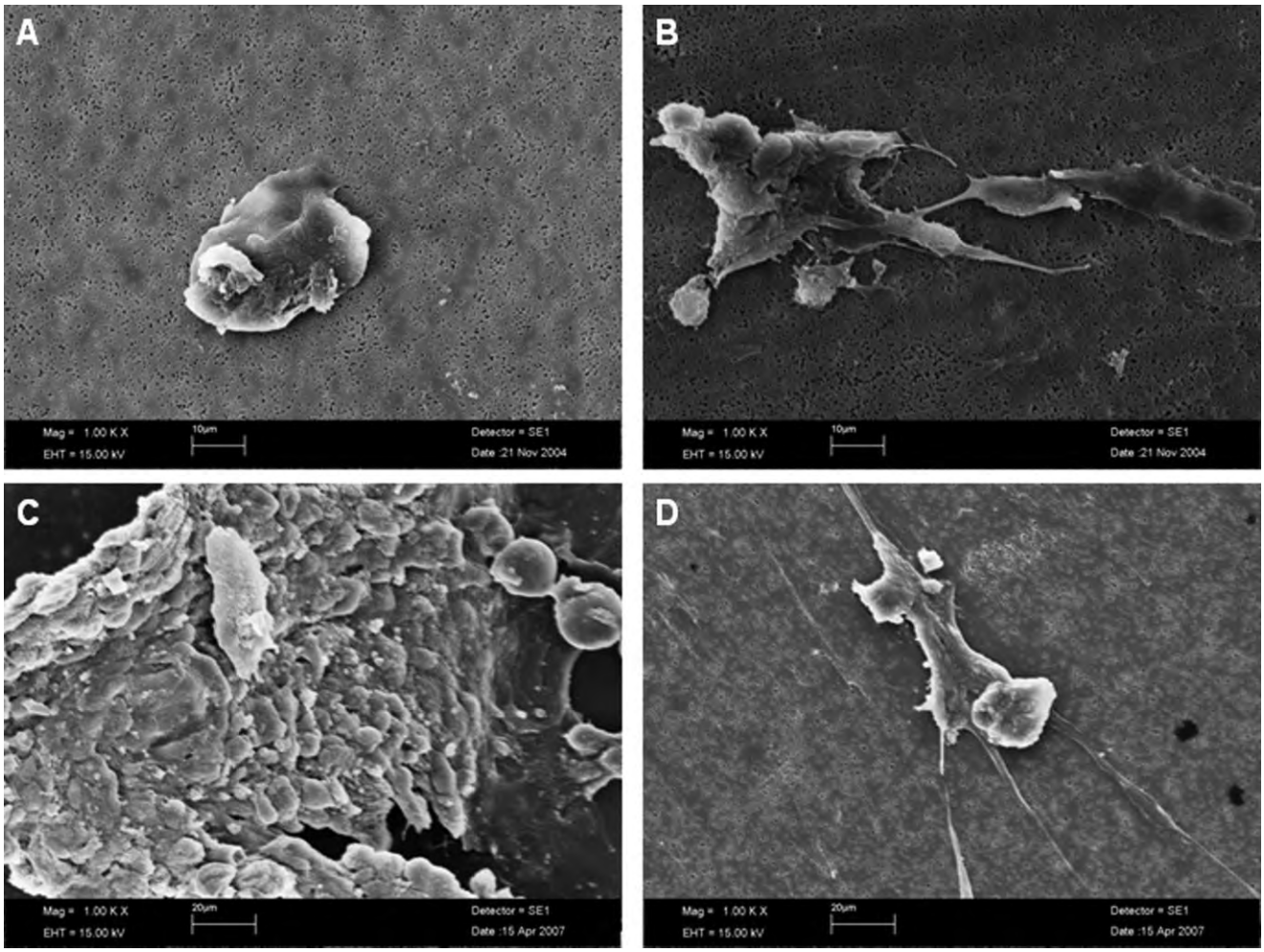

Figure 5. Scanning electron microscopy of polycarbonate membrane seeded with human oral keratinocytes and human osteoblast-like cells after 1 week (A, B) and after 2 weeks (C, D) under static culture conditions. Typical appearance of oral keratinocytes (A, C) and osteoblast-like cells (B, D). Magnification ×1000.

On collagen membranes confluent and multilayered cell growth was detected more often when compared with polycarbonate membranes (Figs. 7 and 8). On most of the collagen membranes confluent, partly multilayered growth was visible after a cultivation period of 1 week in the incubator [Fig. 7(A,B)]. After a cultivation period for 2 weeks, the cell density of keratinocytes and osteoblasts increased [Fig. 7(C,D)].

Confluent growth of osteoblast-like cells was visible on collagen membranes after a cultivation period of 1 week in the perfusion chamber [Fig. 8(B)]. Oral keratinocytes were not as spread as after cultivation under static conditions [Fig. 8(A)]. After a cultivation period of 2 weeks in perfusion chamber on all collagen membranes, confluent and more dense growth of osteoblast-like cells [Fig. 8(D)] was visible, whereas only conglomerates or even singular cells with the aspect of keratinocytes [Fig. 8(C)] on the other side of the membranes were detectable.

Cell characterization

Osteocalcin determination and alkaline phosphatase assay

The characterization of osteoblast-like cells was determined by the amount of osteocalcin and the relative amount of alkaline phosphatase.

The amount of osteocalcin in the supernatant varied between 9.2 and 14.5 ng/mL.

The alkaline staining of these cells typically resulted intensively positive (about 70%; Fig. 9).

Cytokeratine 13 and 19

The characterization of oral keratinocytes was determined by the presence of expressing cytokeratine 13 and 19 (Fig. 10). Immunocytochemistry of the fixed cells showed the presence of cytokeratine

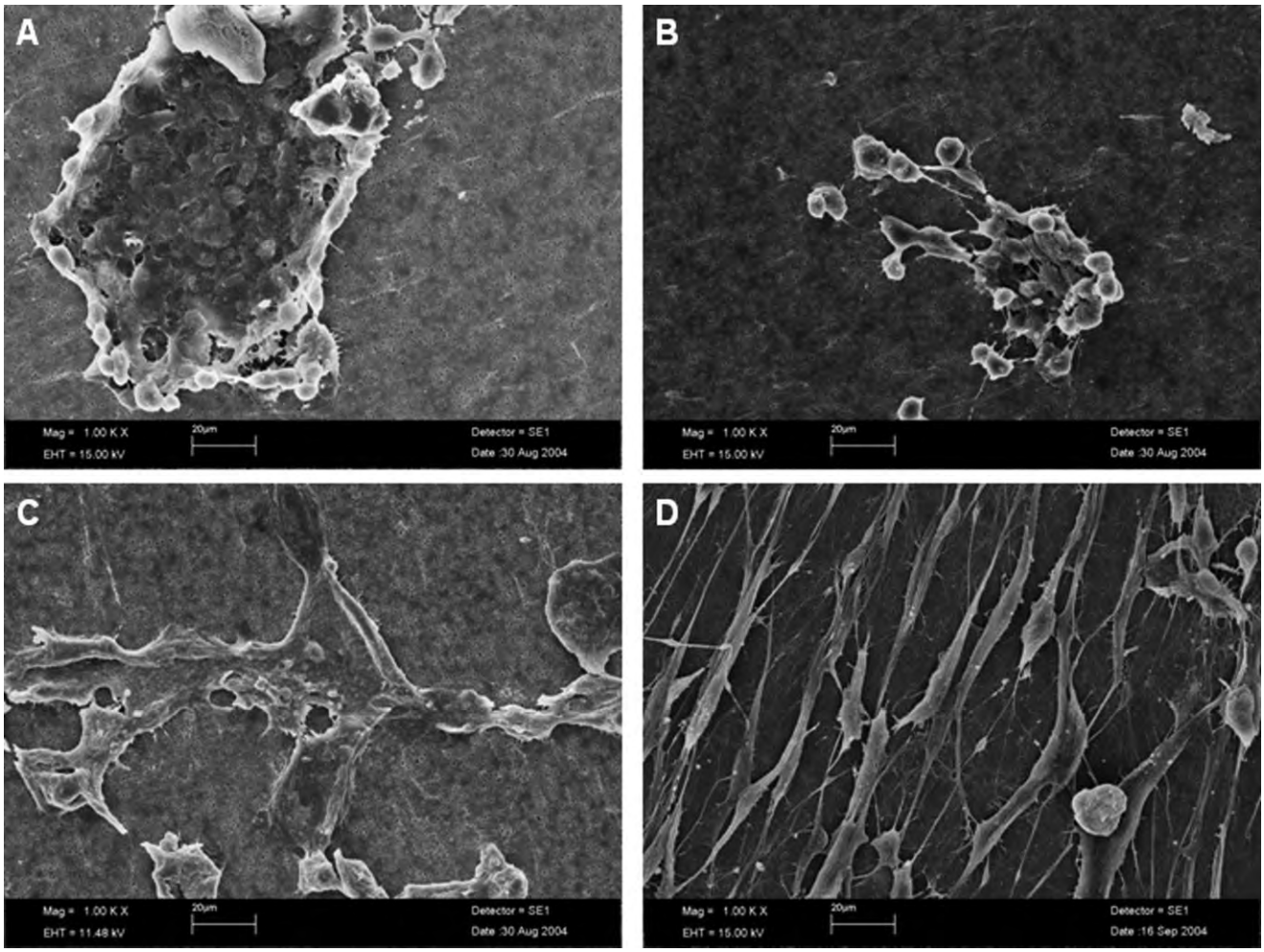

Figure 6. Scanning electron microscopy of a polycarbonate membrane seeded with human oral keratinocytes and human osteoblast-like cells after 1 week (A, B) and after 2 weeks (C, D) in perfusion culture. Typical appearance of oral keratinocytes (A, C) and osteoblast-like cells (B, D). Magnification ×1000.

13 [Fig. 10(A)] as well as cytokeratine 19 in all samples [Fig. 10(B)].

DISCUSSION

Human oral keratinocytes and human osteoblast-like cells are common in tissues involved in head and neck defects. For the later combined reconstruction of hard and soft tissues, the cocultivation of these cells was investigated for the first time. As carriers, laminin-coated polycarbonate membranes and equine collagen membranes were used. The cultivation was performed under static conditions and in perfusion chamber. In this study, we could show that it is possible to cocultivate human oral keratinocytes and human osteoblast-like cells on the two different sides of one biomaterial. According to previous findings of our laboratory, the cells do not support each other but the proliferation seems to be less than expected when compared with the growth of the cultivation of each cell type alone.[30] Up to now, no examination exists concerning the combination of human oral keratinocytes and human osteoblast-like cells.

Cocultivation of different cells was mainly investigated studying skin models, consisting of dermal and epidermal layers to treat full-thickness skin defects.[9–11,31] Rheinwald and Green (1975) described that the growth of dermal kertinocytes is supported by cocultivation with fibroblasts.[32,33] According to this for the cultivation of keratinocytes, a feeder layer of mostly lethally irradiated fibroblasts was used and the effects of cocultivation of these cells even together with adipocytes were investigated.[34–36] In a rat skin model, Sugihara et al. (2001) found that the proliferation and differentiation of keratinocytes was supported by adipocytes but cocultivated fibroblasts were inhibited.[36] Also for the cultivation of oral keratinocytes, the cocultivation with fibroblasts was used.[13,37] Additionally, effects of interactions

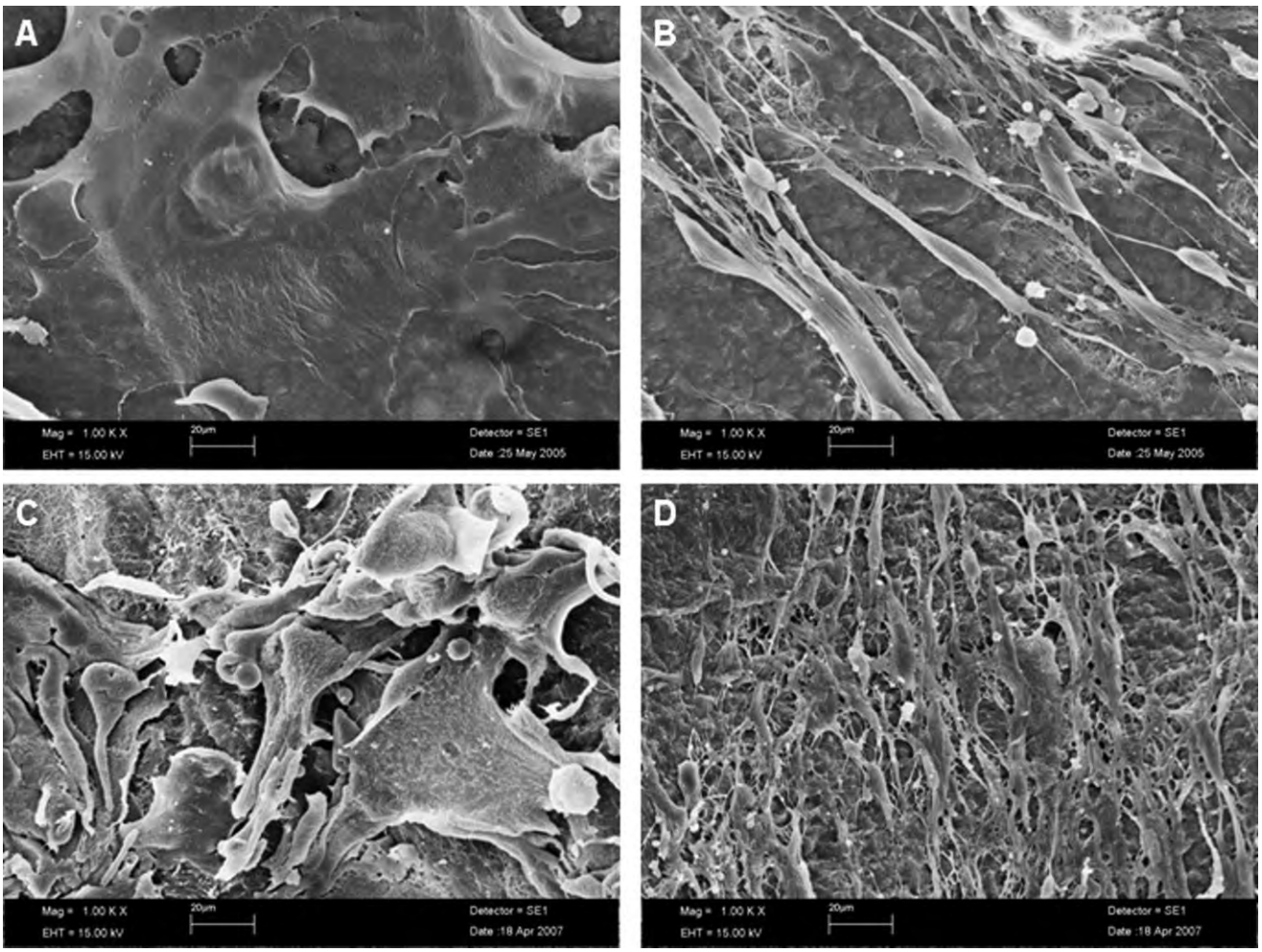

Figure 7. Scanning electron microscopy of collagen membrane seeded with human oral keratinocytes and human osteoblast-like cells after 1 week (A, B) and after 2 weeks (C, D) under static culture conditions. Typical appearance of oral keratinocytes (A, C) and osteoblast-like cells (B, D). Magnification ×1000.

between both cell types were studied.[38] Even if oral and dermal keratinocytes show many similarities, the former seem to be more difficult to cultivate. For example, human oral keratinocytes stop proliferating much earlier under the same culture conditions when compared with dermal keratinocytes.[39] Therefore, it is not possible to transfer results obtained with dermal keratinocytes to oral keratinocytes. This is also be true for studying the interaction with other cell types.

The cultivation of bone was widely investigated in the field of head and neck reconstructions and dental implantology.[5,40] To the best of our knowledge up to now, the combination of oral keratinocytes and osteoblast-like cells to create hard and soft tissue replacements was not described and interactions of osteoblasts and keratinocytes were not examined. Spector et al. (2002) investigated the cocultivation of rat osteoblasts and immature dural cells, which leads to higher proliferation and differentiation of the osteoblasts when compared with osteoblasts cultured alone due to paracrine factors.[41] Maurin et al. (2000) described the inhibition of the proliferation of human bone-derived osteoblasts by cocultivated human adipocytes. For human bone-marrow-derived osteoblast-like cells and human adipocytes, this effect could not be found.[42]

In this study, collagen membranes showed significant better results relating to cell proliferation when compared with laminin-coated polycarbonate membranes although both materials were previously described as suitable carriers for the cultivation of human oral keratinocytes and human osteoblast-like cells.[4,13–15,43,44] One reason for the better cell proliferation rate could be that the attachment of cells on collagen as a typical protein of extracellular matrix is facilitated when compared with the attachment on polycarbonate membranes. Schleicher et al. (2005) described a better attachment of osteoblasts to collagen in comparison to polyglycic acid.[4] Also Webster et al. (2002) explained the better adhesion of osteoblasts to yttrium-pretreated hydroxylapatite (HA)

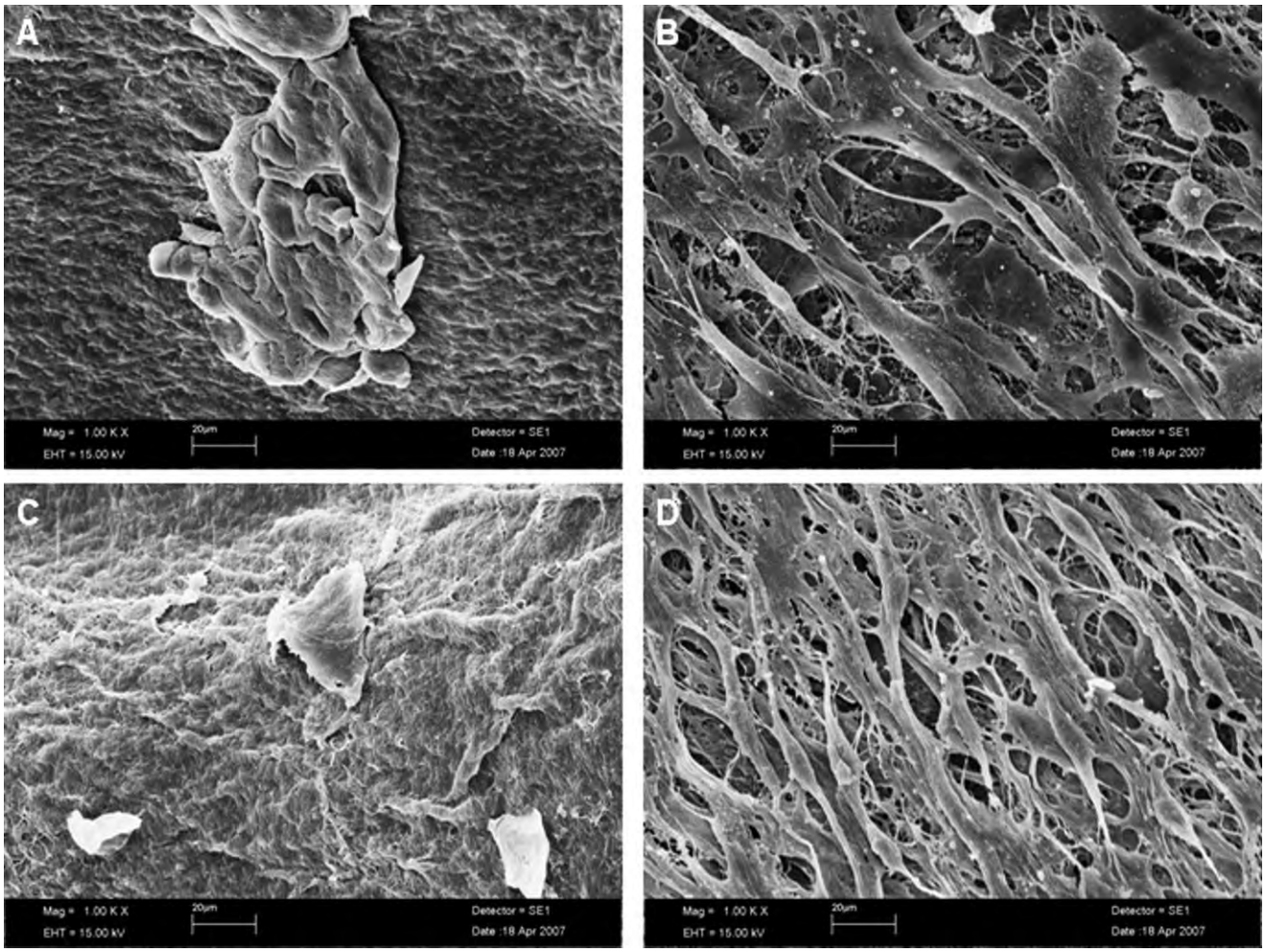

Figure 8. Scanning electron microscopy of collagen membrane seeded with human oral keratinocytes and human osteoblast-like cells after 1 week (A, B) and after 2 weeks (C, D) in perfusion culture. Typical appearance of oral keratinocytes (A, C) and osteoblast-like cells (B, D). Magnification ×1000.

with binding of calcium and following binding of collagen to this material.[45] In our study, the laminin-coating does not support the cells in the same way as collagen membranes do. This is in contrast to other studies which reported that laminin-coating of polycarbonate membranes enhances the growth of osteoblast-like cells and supports the attachment of oral keratinocytes to culture dishes.[17] Another aspect could be the avoidance keratinocyte-osteoblast cell contact because of the thicker collagen membrane. A direct cell contact of the different cell types could lead to a growth inhibition.

In this *in vitro* study, not only different carriers but also different culture conditions (static culture in an incubator and perfusion culture) were compared. Overall after 1 week of cultivation, we could not find any significant differences between the two culture conditions. In contrast, if the cell proliferation after 1 week and after 2 weeks for static culture and perfusion culture were compared, we could find sig-

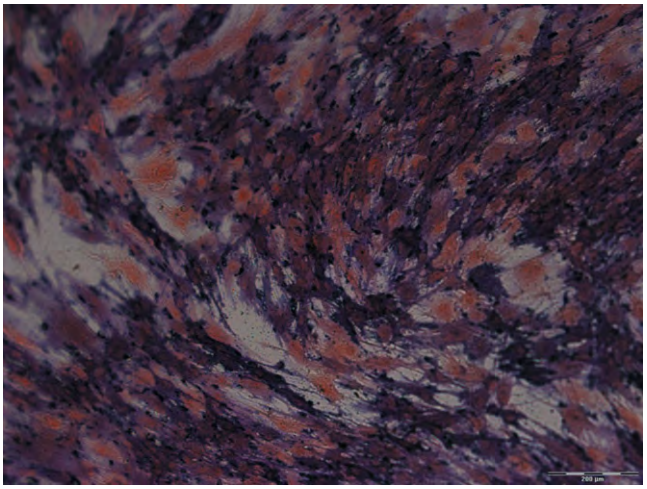

Figure 9. Detection of alkaline phosphatase (blue), alkaline phosphatase negative cells are stained red. Magnification ×10. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

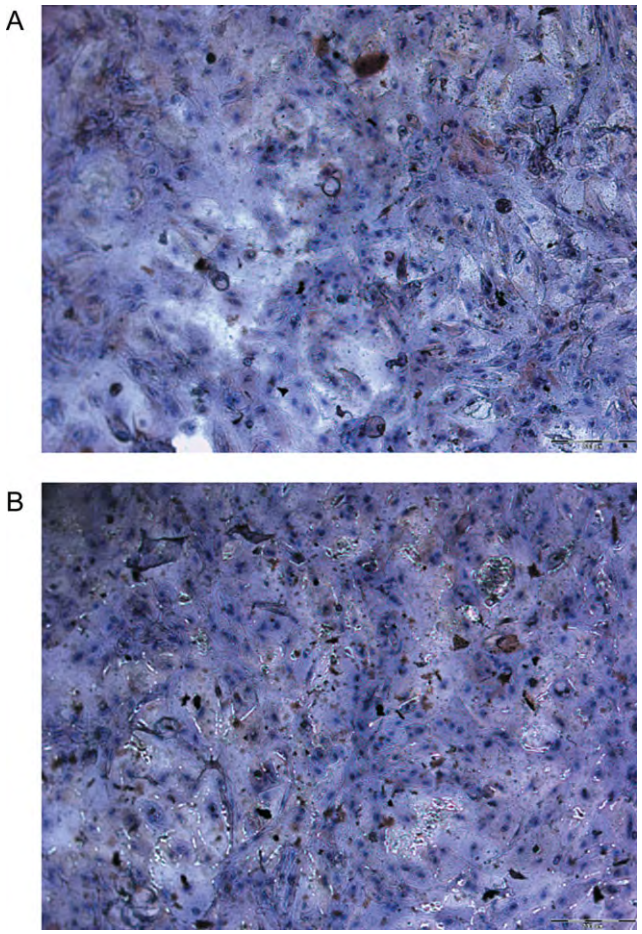

Figure 10. Detection of cytokeratin 13 (A) and cytokeratin 19 (B). Brown cytoplasmatic deposits, nuclei blue. Magnification ×10. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

nificant differences in proliferation over the culture period depending of the culture conditions. After a cultivation period of 2 weeks under static culture conditions, the proliferation of the cells was significantly higher when compared with a cultivation period of 1 week. In the perfusion culture, there were negligible differences in proliferation rates after 1 and 2 weeks of cultivation. These results seem to be in contrast to the fact that a perfusion culture system is able to guarantee the continuous exchange of medium and constant removal of metabolic waste which was shown to have a positive influence on the growth of osteoblast-like cells as well as of oral keratinocytes.[18,22–24] But if we take into consideration that perfusion culture should guarantee more organotypic conditions than static culture and that perfusion culture is used to generate or maintain differentiated tissues.[19,20,25,46] proliferation should be lower when compared with static conditions which do not stimulate the cells to differentiate. Minuth et al. (1990) introduced the system for the cultivation of renal epithelia and Kloth et al. (1994) could show that developing renal microvasculature can be maintained under perfusion culture.[47,48] There are investigations to maintain gastric epithelium under perfusion culture which would be dissolved under static conditions.[19] Additionally, for the long term cultivation of auricular cartilage and human gingiva perfusion culture is shown to be appropriate.[25,49] The tissues keep their typical appearance and biochemical qualities. One aspect for the differentiation of osteoblast-like cells is fluid flow mediated shear stress.[18,26–29] This aspect supports our findings because differentiated cells do not proliferate to the same extent than dedifferentiated cells do.[20,25] After 2 weeks of perfusion culture, less keratinocytes were visible by scanning electron microscopy when compared with a cultivation period of 1 week, whereas osteoblasts were even increased in cell number. This could be due to the facilitated detachment of cells in perfusion culture caused by the flow rate when compared with the cultivation under static conditions, especially when oral keratinocytes have reached their maximum life span. The life span of oral keratinocytes is much shorter than the life span of dermal keratinocytes when cultured under the same conditions.[39] Detached cells could be also a reason for lower results in the proliferation test. Additionally, in perfusion culture osteoblast-like cells proliferate more when compared with oral keratinocytes.[30] This could lead to a growth inhibition of oral keratinocytes. In experiments of our laboratory, it could be demonstrated that osteoblast-like cells and oral keratinocytes grow better if they are separated by a barrier.[30]

In conclusion, for the first time the cocultivation of human oral keratinocytes and human osteoblast-like cells is feasible. Collagen membranes are more advantageous than polycarbonate membranes even if the latter are coated with laminin. Furthermore, under perfusion culture conditions the cell proliferation does not increase over time. This could be attributed to differentiation of the cells or the loss of cells after reaching their maximum life span or because of the inhibition of oral keratinocytes by the more expanding osteoblast-like cells. The examination of cellular differentiation under perfusion culture conditions in comparison to static culture will be subject of further investigations. Additionally, these *in vitro* results are necessary to provide a basis for planned *in vivo* studies (animal model) to reconstruct craniomaxillofacial defects.

References

1. Unger RE, Huang Q, Peters K, Protzer D, Paul D, Kirkpatrick CJ. Growth of human cells on polyethersulfone (PES) hollow fiber membranes. Biomaterials 2005;26:1877–1884.

2. Stock UA, Vacanti JP. Tissue engineering: Current state and prospects. Annu Rev Med 2001;52:443–451.
3. Wan DC, Nacamuli RP, Longaker MT. Craniofacial bone tissue engineering. Dent Clin North Am 2006;50:175–190, vii.
4. Schleicher I, Parker A, Leavesley D, Crawford R, Upton Z, Xiao Y. Surface modification by complexes of vitronectin and growth factors for serum-free culture of human osteoblasts. Tissue Eng 2005;11:1688–1698.
5. Schmelzeisen R, Schimming R, Sittinger M. Making bone: Implant insertion into tissue-engineered bone for maxillary sinus floor augmentation-a preliminary report. J Craniomaxillofac Surg 2003;31:34–39.
6. Lauer G, Schimming R, Gellrich NC, Schmelzeisen R. Prelaminating the fascial radial forearm flap by using tissue-engineered mucosa: Improvement of donor and recipient sites. Plast Reconstr Surg 2001;108:1564–1572; discussion 1573–1575.
7. Schultze-Mosgau S, Lee BK, Ries J, Amann K, Wiltfang J. In vitro cultured autologous pre-confluent oral keratinocytes for experimental prefabrication of oral mucosa. Int J Oral Maxillofac Surg 2004;33:476–485.
8. Neovius EB, Kratz G. Tissue engineering by cocultivating human elastic chondrocytes and keratinocytes. Tissue Eng 2003;9:365–369.
9. Cooper ML, Andree C, Hansbrough JF, Zapata-Sirvent RL, Spielvogel RL. Direct comparison of a cultured composite skin substitute containing human keratinocytes and fibroblasts to an epidermal sheet graft containing human keratinocytes on athymic mice. J Invest Dermatol 1993;101:811–819.
10. Hansbrough JF, Morgan JL, Greenleaf GE, Bartel R. Composite grafts of human keratinocytes grown on a polyglactin mesh-cultured fibroblast dermal substitute function as a bilayer skin replacement in full-thickness wounds on athymic mice. J Burn Care Rehabil 1993;14:485–494.
11. Bannasch H, Momeni A, Knam F, Stark GB, Fohn M. Tissue engineering of skin substitutes. Panminerva Med 2005;47:53–60.
12. Tomakidi P, Breitkreutz D, Fusenig NE, Zoller J, Kohl A, Komposch G. Establishment of oral mucosa phenotype in vitro in correlation to epithelial anchorage. Cell Tissue Res 1998;292:355–366.
13. Tomakidi P, Fusenig NE, Kohl A, Komposch G. Histomorphological and biochemical differentiation capacity in organotypic co-cultures of primary gingival cells. J Periodontal Res 1997;32:388–400.
14. Lauer G, Otten JE. Cultivation of gingival keratinocytes on permeable membranes: Simulation of the function of mouth cavity epithelium. Mund Kiefer Gesichtschir 1997;1:35–38.
15. Wiedmann-Al-Ahmad M, Gutwald R, Lauer G, Hubner U, Schmelzeisen R. How to optimize seeding and culturing of human osteoblast-like cells on various biomaterials. Biomaterials 2002;23:3319–3328.
16. Ignatius A, Blessing H, Liedert A, Schmidt C, Neidlinger-Wilke C, Kaspar D, Friemert B, Claes L. Tissue engineering of bone: Effects of mechanical strain on osteoblastic cells in type I collagen matrices. Biomaterials 2005;26:311–318.
17. Lauer G, Siegmund C, Hubner U. Influence of donor age and culture conditions on tissue engineering of mucosa autografts. Int J Oral Maxillofac Surg 2003;32:305–312.
18. Wang Y, Uemura T, Dong J, Kojima H, Tanaka J, Tateishi T. Application of perfusion culture system improves in vitro and in vivo osteogenesis of bone marrow-derived osteoblastic cells in porous ceramic materials. Tissue Eng 2003;9:1205–1214.
19. Kloth S, Eckert E, Klein SJ, Monzer J, Wanke C, Minuth WW. Gastric epithelium under organotypic perfusion culture. In Vitro Cell Dev Biol Anim 1998;34:515–517.
20. Minuth WW, Sittinger M, Kloth S. Tissue engineering: Generation of differentiated artificial tissues for biomedical applications. Cell Tissue Res 1998;291:1–11.
21. Sittinger M, Bujia J, Minuth WW, Hammer C, Burmester GR. Engineering of cartilage tissue using bioresorbable polymer carriers in perfusion culture. Biomaterials 1994;15:451–456.
22. Cartmell SH, Porter BD, Garcia AJ, Guldberg RE. Effects of medium perfusion rate on cell-seeded three-dimensional bone constructs in vitro. Tissue Eng 2003;9:1197–1203.
23. van den Dolder J, Bancroft GN, Sikavitsas VI, Spauwen PH, Jansen JA, Mikos AG. Flow perfusion culture of marrow stromal osteoblasts in titanium fiber mesh. J Biomed Mater Res 2003;64A:235–241.
24. Navarro FA, Mizuno S, Huertas JC, Glowacki J, Orgill DP. Perfusion of medium improves growth of human oral neomucosal tissue constructs. Wound Repair Regen 2001;9:507–512.
25. Strehl R, Tallheden T, Sjogren-Jansson E, Minuth WW, Lindahl A. Long-term maintenance of human articular cartilage in culture for biomaterial testing. Biomaterials 2005;26:4540–4549.
26. Bancroft GN, Sikavitsas VI, van den Dolder J, Sheffield TL, Ambrose CG, Jansen JA, Mikos AG. Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. Proc Natl Acad Sci USA 2002;99:12600–12605.
27. Sikavitsas VI, Bancroft GN, Holtorf HL, Jansen JA, Mikos AG. Mineralized matrix deposition by marrow stromal osteoblasts in 3D perfusion culture increases with increasing fluid shear forces. Proc Natl Acad Sci USA 2003;100:14683–14688.
28. Reich KM, Gay CV, Frangos JA. Fluid shear stress as a mediator of osteoblast cyclic adenosine monophosphate production. J Cell Physiol 1990;143:100–104.
29. Bakker AD, Soejima K, Klein-Nulend J, Burger EH. The production of nitric oxide and prostaglandin E(2) by primary bone cells is shear stress dependent. J Biomech 2001;34:671–677.
30. Sendic M. Wachstumsverhalten und gegenseitige Beeinflussung von Gingivakeratinozyten und Osteoblasten auf Kollagen. Inaugural-Dissertation zur Erlangung des Zahnmedizinischen Doktorgrades der Medizinischen Fakultät der Albert-Ludwigs-Universität Freiburg i. Br. 2005.
31. Kim BM, Suzuki S, Nishimura Y, Um SC, Morota K, Maruguchi T, Ikada Y. Cellular artificial skin substitute produced by short period simultaneous culture of fibroblasts and keratinocytes. Br J Plast Surg 1999;52:573–578.
32. Rheinwald JG, Green H. Serial cultivation of strains of human epidermal keratinocytes: The formation of keratinizing colonies from single cells. Cell 1975;6:331–343.
33. Rheinwald JG, Green H. Formation of a keratinizing epithelium in culture by a cloned cell line derived from a teratoma. Cell 1975;6:317–330.
34. Green H, Rheinwald JG, Sun TT. Properties of an epithelial cell type in culture: The epidermal keratinocyte and its dependence on products of the fibroblast. Prog Clin Biol Res 1977;17:493–500.
35. Sugihara H, Toda S, Miyabara S, Kusaba Y, Minami Y. Reconstruction of the skin in three-dimensional collagen gel matrix culture. In Vitro Cell Dev Biol 1991;27A:142–146.
36. Sugihara H, Toda S, Yonemitsu N, Watanabe K. Effects of fat cells on keratinocytes and fibroblasts in a reconstructed rat skin model using collagen gel matrix culture. Br J Dermatol 2001;144:244–253.
37. Formanek M, Millesi W, Willheim M, Scheiner O, Kornfehl J. Optimized growth medium for primary culture of human oral keratinocytes. Int J Oral Maxillofac Surg 1996;25:157–160.
38. Gron B, Stoltze K, Andersson A, Dabelsteen E. Oral fibroblasts produce more HGF and KGF than skin fibroblasts in response to co-culture with keratinocytes. APMIS 2002;110:892–898.

39. Kirschner M, Montazem A, Hilaire HS, Radu A. Long-term culture of human gingival keratinocyte progenitor cells by down-regulation of 14-3-3 sigma. Stem Cells Dev 2006; 15:556–565.
40. Warnke PH, Springer IN, Wiltfang J, Acil Y, Eufinger H, Wehmoller M, Russo PA, Bolte H, Sherry E, Behrens E, Terheyden H. Growth and transplantation of a custom vascularised bone graft in a man. Lancet 2004;364:766–770.
41. Spector JA, Greenwald JA, Warren SM, Bouletreau PJ, Crisera FE, Mehrara BJ, Longaker MT. Co-culture of osteoblasts with immature dural cells causes an increased rate and degree of osteoblast differentiation. Plast Reconstr Surg 2002;109:631–642; discussion 643–644.
42. Maurin AC, Chavassieux PM, Frappart L, Delmas PD, Serre CM, Meunier PJ. Influence of mature adipocytes on osteoblast proliferation in human primary cocultures. Bone 2000;26:485–489.
43. Lauer G, Schimming R. Tissue-engineered mucosa graft for reconstruction of the intraoral lining after freeing of the tongue: A clinical and immunohistologic study. J Oral Maxillofac Surg 2001;59:169–175; discussion 175–177.
44. Wenger A, Stahl A, Weber H, Finkenzeller G, Augustin HG, Stark GB, Kneser U. Modulation of in vitro angiogenesis in a three-dimensional spheroidal coculture model for bone tissue engineering. Tissue Eng 2004;10:1536–1547.
45. Webster TJ, Ergun C, Doremus RH, Bizios R. Hydroxylapatite with substituted magnesium, zinc, cadmium, and yttrium. II. Mechanisms of osteoblast adhesion. J Biomed Mater Res 2002;59:312–317.
46. Minuth WW, Strehl R, Schumacher K, de Vries U. Long term culture of epithelia in a continuous fluid gradient for biomaterial testing and tissue engineering. J Biomater Sci Polym Ed 2001;12:353–365.
47. Minuth WW, Rudolph U. A compatible support system for cell culture in biomedical research. Cytotechnology 1990;4: 181–189.
48. Kloth S, Schmidbauer A, Kubitza M, Weich HA, Minuth WW. Developing renal microvasculature can be maintained under perfusion culture conditions. Eur J Cell Biol 1994; 63:84–95.
49. Lehmann P, Kloth S, Aigner J, Dammer R, Minuth W. Vital long-term preservation of human gingiva in perfusion culture. Mund Kiefer Gesichtschir 1997;1:26–30.