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Search for ideal biomaterials to cultivate human osteoblast-like cells for reconstructive surgery

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In this study we cultured human osteoblast-like cells on 16 different biomaterials to find an optimal biomaterial for subsequent use in reconstructive surgery. The tested biomaterials can be divided into five groups: collagen-based membranes of bovine, equine or calf origin, tricalcium phosphate based membranes (alpha and beta), hyaluronic acid based, anorganic bovine bone and anorganic silicone-based membranes. Cell proliferation and cell colonization (**E**nvironmental **S**canning **E**lectron **M**icroscope, ESEM) analysis were performed.

The results of the study demonstrated that four of the examined biomaterial/cell constructs showed a very good proliferation rate and cell density: No. 3 (Tissue Vlies®), No. 7 (Sepra film), No. 16 (Biobrane®) and No. 17 (Biomend™). No favourable group of biomaterials was noticeable. Moreover, the results indicate that these four biomaterials as a part of bone constructs are the best tools for engineering new bone tissue. In contrast, biomaterials No. 19a (Bio-Oss®) and 19b (Bio-Oss® Collagen) showed the lowest proliferation rates. The result of No. 19b was improved by treatment in the perfusion chamber for 48 h as well as by additional use of vacuum. The present study is an important base for further analysis of biomaterials and consequentely for the development of tissue engineering.

1. Introduction

Until now, osseous defects are treated with autologous bone with the disadvantage that the bone graft is limited. Additionally, a second surgical procedure is necessary which frequentely implies the risk of donor site morbidity. Complications like pains at the removal site over six months, infections, haematoma, disorders in the sensitivity and fractures were observed [1–4]. Recently, new methods were developed to avoid these disadvantages, especially the use of different biomaterials. In general, two different types of biomaterials for tissue regeneration exists: nonresorbable and bioresorbable membranes. These commercially available cell carrier matrices differ in composition, pore size and pore density, as well as in permeability and durability [5]. The first commercially available biomaterials were made of polytetrafluor-ethylene (Gore-Tex) and were nonresorbable [6]. In consequence they still have the disadvantage that they have to be removed. For tissue engineering resorbable membranes are of main interest. Moreover, such resorbable membranes demands that they are not cytotoxic and show an optimal osseous integration. It was recently reported that wounds treated with biomaterials in combination with cultured cells shows a better regeneration as biomaterials alone [7]. The cells serves among other things as donors for natural growth factors and cytokines. Consequentely, the cells and for our study the osteoblasts have to be capable of growing in a three dimensional structure for subsequent transplantation as well as for following bone regeneration. Up to now, the search for the perfect biomaterial for tissue engineered bone formation still continues. Studies exist about the growth of osteoblasts with different origin and with single biomaterials. Lee *et al.* [8] studied the growth of fetal rat calvarial osteoblastic cells on chitosan/tricalcium phosphate sponges as tissue engineered scaffolds for bone formation, Piattelli *et al.* [9] used anorganic bovine bone (Bio-Oss®) in sinus augmentation procedures and Alpar *et al.* [6] used polylactic acid (Guidor) as well as collagen type I and III (Bio-Gide) for the growth of primary human peridontal ligament fibroblast and human osteogenic sarcoma cells. Also animal studies exist comparing the biological behaviour of biomembranes such as the study of Cancian *et al.* [10] who used the biomaterials BioGran and Calcitite as fillers

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for surgical cavities in the mandibles of adult monkeys. In the study of Saad *et al.* [11] a polyesterurethane foam (DegraPol) as degradable carrier for bone morphogenetic proteins was implanted subcutaneously into rats. Weng *et al.* [12] seeded osteoblasts on a polymer template composed of polyglycolic acid and polylactic acid and implanted this construct into subcutaneous pockets at the dorsum of athymic mice. Interestingly, no study exist about the growth of human osteoblast-like cells on multitude different biomaterials to compare directly the cell colonization and attachment and consequentely to get knowledge about the ideal biomaterial which can be used as bone substitute in reconstructive procedures. Our recent study [13] examined the seeding and cultivation procedure in order to optimize the attachment and growth of human osteoblasts on three various cell carriers (a native bovine collagen membrane, a native equine collagen membrane and a native calf collagen membrane). The best proliferation rate was achieved with 2 h adding the culture medium after initial seeding, a seeding density of 1×10^5 cells/ml and with the bovine collagen membrane (Tissue Vlies®).

In our present study we focus on the growth of human osteoblast-like cells on 16 various resorbable biomaterials. The biomaterials investigated in this study were of different composition and different surface morphologies. All biomaterials were biocompatible. The tested biomaterials can be divided into five groups: collagen-based membranes of bovine, equine or calf origin, tricalcium phosphate based membranes (alpha and beta), hyaluronic acid based, anorganic bovine bone membranes and an anorganic silicone-based membrane.

The aim of this study was to find the ideal biomaterial for subsequent use in reconstructive and bone surgery. The creation of such optimal autologous grafts would be a further development of tissue engineering.

2. Materials and methods

2.1. Biomaterials

Five different groups of 16 biomaterials in all were used for the cultivation of human osteoblast-like cells and for potential subsequent grafting; all investigated commercially available materials can be used as tissue/bone substitute, for augmentation and reconstruction of defects:

1. Collagen-based membranes

All collagen-based membranes used in this study are resorbable. The three-dimensional matrix allows for the integration of connective tissue flaps as well as passage of essential nutrients. The contact of collagen with blood results in the aggregation of thrombocytes which then adhere in great numbers to the collagen matrix, disintegrate and release coagulation factors which, in combination with plasma factors, lead to the formation of fibrin.

- native bovine collagen membrane: Tissue Vlies®, Baxter, Heidelberg, Germany (No. 3), 1 cm^2 contains 2.8 mg collagen, pore size variable
- native bovine collagen membrane: Tissue Fascie®, Immuno, Heidelberg, Germany (No. 6), 1 cm^2 contains 4.0 mg collagen, pore size variable
- pure bovine collagen type I membrane from the achilles‘ tendon: Biomend™, Sulzer Calcitek, Carlsbad, USA (No. 17), pore size 0.004 μm
- native equine collagen membrane from the achilles’ tendon: Resorba®, Nürnberg, Germany (No. 13), eliminated from all non-collagenous components, pore size 200 μm in average
- native equine collagen membrane: Tissue Foil E®, Nürnberg, Germany (No. 21), 1 cm^2 contains 4.0 mg collagen, none other ingredients, pore size variable
- native calf collagen membrane Osteovit®, B. Braun-Dexon GmbH, Spangenberg, Germany (No. 14), the porous collagen structure encourages tissue penetration, it consists of spongiosa eliminated from antigenes, enzymes, fatty acids, minerals and all not-collagen compositions by a special treatment. 1 cm^3 implant material contains 1 cm^3 spongiosa
- native pig collagen membrane: Bio-Gide®, Geistlich Biomaterials, Baden- Baden, Germany (No. 18). Bio-Gide® has a bilayer structure: The porous surface will allow the ingrowth of bone forming cells and the dense surface will prevent the ingrowth of fibrous tissue into the bone defect. The membrane is made of collagen type I and type II without further crosslinking or chemical treatment.

2. Tricalciumphosphate-based membranes

- alpha-tricalcium phosphate membrane: BioBase®, Sulzer Calcitek, Carlsbad, USA with a pore size of 0.5–1.4 mm (No. 1), roentgenspectrometrical analysis showed that this material is nearly monophasic
- beta-tricalcium phosphate ceramic (tertiary calcium salt of orthophosphoric acid) membrane: Bio Resorb, Oraltronics, Ilmenau, Germany, fine-grained (No. 22a); the analytical composition is as follows (mass-%): calcium oxide 52.0–54.2, phosphor pentoxide 45.8–48.0. The material has a high interconnective porosity. The Ca/P atom ratio is 1.5 which is similar to the Ca/P-atom ratio of the mineral phase of the natural bone, bioresorbable
- beta-tricalcium phosphate membrane: Bio Resorb, Oraltronics, Ilmenau, Germany, rough-grained (No. 22b), components according to type and quantity see No. 22a, bioresorbable
- carbonapatite membrane: Norian SRS (sceletal repair system), Cupertino, USA (No. 8). Norian SRS is an injectable calcium phosphate cement which hardens in situ and cures by a cristallization reaction to form dahllite, a carbonated apatite, equivalent to bone mineral. The Ca/P atom ratio is 1.67, which is similar to the mineral phase of the natural bone. The cristallization dimension comes to 204 Å. The material

is gradually remodelled by osteoclasts and osteoblasts.

3. Hyaluronic acid-based membranes

   – chemical modified sodium-hyaluronic acid and carboxymethylcellulose membrane: Sepra film, Genzyme, Lübeck (No. 7). This membrane is an adhesion barrier. Glycerin is added to improve the flexibility of the membrane. The membrane is approved for use in open abdominal and pelvic procedures and it is slowly resorbed into the body
   – hyaluronic acid ester: Jaloskin®, MARKA GmbH, Frankfurt, Germany (No. 11) is a natural occuring biodegradable extracellular matrix molecule

4. Anorganic bovine bone membrane

   – deproteinized sterilized bovine bone with 75% to 80% porosity and with a crystal size of approximately 10 nm in the form of cortical and cancellous blocks [14]: Bio-Oss®, Geistlich Biomaterials, Baden-Baden, Germany (No. 19a). Bio-Oss® is a natural bone mineral of bovine origin which is produced in a multistage purification process. The mineralized bone structure is largely chemically comparable with mineralized human bone (similar macro- and microscopic structure to human spongiosa bone). The cristallization dimension comes to 400–1000 Å. The inner surface area of the material is approximately 100 m^2/g, wide interconnecting pore system. Bio-Oss®can be gradually remodelled by osteoclasts and osteoblasts
   – combination of 100 mg spongiosa granules and 10% collagen fibres in a block form: Bio-Oss® Collagen, Geistlich Biomaterials, Baden-Baden, Germany (No. 19b). The collagen provides the fixation of the Bio-Oss® at the desired place and is slowly resorbed an replaced by new bone cells.

     Additonally, Bio-Oss® Collagen was stored in the perfusion chamber for 48 h in 500 ml cell culture medium (Opti-MEM, Gibco Laboratories Life Technologies, Inc, Grand Island, NY, USA; No. 19c) as well as in the perfusion chamber followed by the use of vacuum for 5 min (No. 19 d).

5. Anorganic silicone-based membrane

   – ultrathin, semipermeable silicone membrane mechanically bonded to a flexible knitted trifilament nylon fabric: Biobrane®, Bertek Pharmaceuticals Inc. , Sugar Land, Texas, USA (No. 16). A nontoxic mixture of highly purified peptides derived from porcine dermal collagen has been bonded to the nylon/silicone membrane to provide a highly flexible and conformable composite dressing with adherence properties and a hydrophilic, biocompatible surface. Pore size 500 μm in average.

2.2. Isolation and cultivation of human osteoblast-like cells

For studying the growth of human osteoblast-like cells on porous biomaterials, human bone cells were isolated from cortico-lamellar bone of the maxilla during biopsies. The biopsies from ten different patients of each sex in the age of 23–65 years were crumbled into explants (size 2 mm × 2 mm) and seeded on culture flasks (25 cm^2, Greiner, Frickenhausen, Germany) using Opti-minimal essential medium (Opti-MEM, Gibco Laboratories Life Technologies, Inc, Grand Island, NY, USA) pH 7.2 with 10% foetal calf serum (FCS) and kept in a humidified atmosphere of 5% CO_2 at 37 °C (Heraeus, Hanau, Germany).

The osteoblast-like cells which migrated onto the floor of the culture dish form a confluent layer after 4–5 weeks (primary culture) and the first passage was used for the growth experiments on the various biomaterials.

2.3. Preparation of the cell culture plates

Before cell seeding onto the biomaterials the 24 well culture plates (Costar, NY, USA) were humidified with culture medium. Of each plasma sterilized biomaterial pieces of 1 cm^2 were placed into three wells: one for scanning electronic microscopic study, one for alkaline phosphatase assay and one for the type I collagen determination. For the proliferation test smaller samples of the three materials were placed in 96 well plates (Corning, NY, USA): one well for the medium blank, one well with the biomaterial only, and three wells with the biomaterial and the seeded cells.

2.4. Cell seeding onto biomaterials

The confluent osteoblast cultures (primary culture) were detached from the culture flask by incubation with 0.5% trypsin (Gibco, Paisley, Scotland) in phosphate buffered saline (PBS) for 8 min at 37 °C. The bone cell solution was filtered through a 100 μm cell-strainer (Falcon, Heidelberg, Germany) in a 50 ml tube (Falcon, Heidelberg, Germany), centrifuged (Biofuge Strato, Heraeus, Hanau, Germany, 1120 × g, 12 min, 30 °C) and resuspended in 1 ml culture medium. The cells were transfered in a 75 cm^2 culture flask (Greiner, Frickenhausen, Germany), filled up with 25 ml culture medium. After 14 days the cells of the first passage were detached again from the culture flask with 0.5% trypsin,centrifugated and resuspended in 1 ml culture medium. After staining with trypanblue (1:1;v/v) the cells still alive were counted in a chamber by light microscopy (Zeiss Axiovert 135, Jena, Germany). Then 100 μl of 10^5 cells/ml were seeded onto the 16 different biomaterials with a sterile syringe. The cells were incubated for 2 h at 37 °C in 5% CO_2 atmosphere before culture medium to a total volume of 1 ml was added. After one week of incubation in total, scanning electron microscopic studies and proliferation assays followed.

Additionally, plates with 1×10^5 cells/ml were incubated for one week at 37 °C in 5% CO_2 atmosphere for the detection of alkaline phophatase and collagen. Once the medium was changed.

2.5. Immunohistochemical stainings

2.5.1. Assay for osteocalcin

For the quantification of osteocalcin in the cell culture supernatant of human maxillar osteoblast-like cells the osteocalcin ELISA (DAKO, Glostrup, Denmark) was performed according to the manufacturer's instructions. In brief, the standards, the curve control and the cell culture supernatants were premixed with biotinylated osteocalcin, incubated in microwells precoated with anti-osteocalcin for 1 h, washed and incubated with peroxidase-conjugated streptavidin for 15 min, which binds strongly to the biotinylated osteocalcin. After a further washing step the chromogenic substrate was added and incubated for 30 min. The reaction was stopped by 2 M H_2SO_4 and the absorbance at 450 nm was measured. Osteocalcin is exclusively synthesized by osteoblasts and is believed to prevent premature mineralization of newly formed, but yet disorganized bone matrix [15].

2.5.2. Assay for alkaline phosphatase and morphometry

For the staining of maxillar osteoblast-like cells an alkaline phosphatase assay kit (Sigma, Deisenhofen, Germany) was used. The culture dishes were air dried, fixed in a citrate-aceton-formaldehyde solution for 30 sec and rinsed gently with Distilled water. Incubation with alkaline phosphatase staining solution for 15 min protected from direct light and a washing step with distilled water followed. The citrate-aceton-formaldehyde solution as well as the alkaline phosphatase staining solution was prepared according to the manufacturer's instructions. The culture dishes were counterstained with neutralred for 5 min, rinsed with Distilled water and dried mounted with cover slips. Positive staining for alkaline phosphatase (red-violet) was identified by lightmicroscopy and evaluated by morphometry using the computer program Analysis 3.1 (Soft Imaging System, Münster, Germany).

2.5.3. Detection of type I collagen

For the quantification of type I collagen the cells were washed in phosphate buffered saline (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 for 5 min. After an incubation of 0.3% H_2O_2 in methanol for 30 min, unspecific immune reactions were blocked with 1% bovine serum albumin for 10 min, before the anti-collagen I antibody (Sigma, Deisenhofen, Germany) was administered for 1 h. An incubation of the biotin-conjugated secondary antibody (Vectastain Elite Kit, Vector Laboratories, Burlingame USA) for 45 min and an incubation of avidin mixed with biotin-conjugated peroxidase (Vectastain Elite Kit, Vector Laboratories, Burlingame USA) for 30 min followed. Sections were rinsed between each incubation step three times with PBS for 5 min. The immunreaction was developed by diaminobenzidine-solution (0.05 mg/l DAB/0.05 M Tris-HCl pH 7.3/0.01% H_2O_2) at room temperature. The sections were counterstained with hematoxylin (Merck, Darmstadt, Germany) for 10 s and mounted in 40% glycerin (Merck, Darmstadt, Germany) in PBS. The evaluation was done by lightmicroscopy and the computer program Analysis 3.1 (Soft Imaging System, Münster, Germany).

2.6. Cell proliferation analysis

For cell proliferation analysis the nonradioactive assay EZ4U –EASY FOR YOU (Biozol diagnostica GmbH; Eching, Germany) was used. This method is based on the finding that living cells are capable of reducing slightly yellow coloured tetrazolium salts to intense red coloured formazan derivates by an intracellular reduction system, mostly located in the mitochondria [16]. These formazan derivates are excreted into the culture medium and the absorbance can be measured with a microplate reader. The amount of coloured formazan derivates correlates with the amount of living cells in the sample. The proliferation assay was carried out according to the manufacturers instructions. As control, human osteoblast-like cells which were grown without any biomaterial were used.

2.7. Cell colonization analysis

The cell colonization analysis was assessed by scanning electron microscopy after a culture period of one week. For scanning electron microscopy the samples were fixed in 4% paraformaldehyde for 2 h at room temperature and incubated in 8% formaldehyde for 2 days at 4 °C. The samples were dehydrated in graded alcohol (30, 50, 70, 80 and 90%, each one time and two times in 99.8%). After critical point drying (CPD 030 Baltec, Wallruf, Germany), according to standard procedure using liquid carbon dioxide, the samples were sputtered with goldpalladium (Plano, Germany) in the SCD 040 (Balzers Union, Wallruf, Germany). The probes were examined via Environmental Scanning Electron-Microscope (ESEM, Electroscan 2020 Philips, Netherlands) at about 25 KV, high vacuum mode. Images were digitized.

As control, human osteoblast-like cells seeded onto glass slides were used.

3. Results

In this study 16 different biomaterials were used as a matrix for human osteoblast-like cells which were isolated from cortico-lamellar bone of the maxilla. These cells showed typical osteoblast-like behaviours, determined by the amount of osteocalcin, the relative amount of alkaline phosphatase activity and the relative amount of the presence of cells expressing type I collagen. Gingival keratinocytes were used as control. All cells used in this study expressed a high amount of osteocalcin (12.22 μg/l) and a high alkaline phosphatase activity (about 64% of the cells) whereas the human gingival keratinocytes did not. The isolated and cultivated cells were also able to produce type I collagen in about 71% of the cells.

The vitality and the proliferation capacity of the osteoblast-like cells seeded onto the different

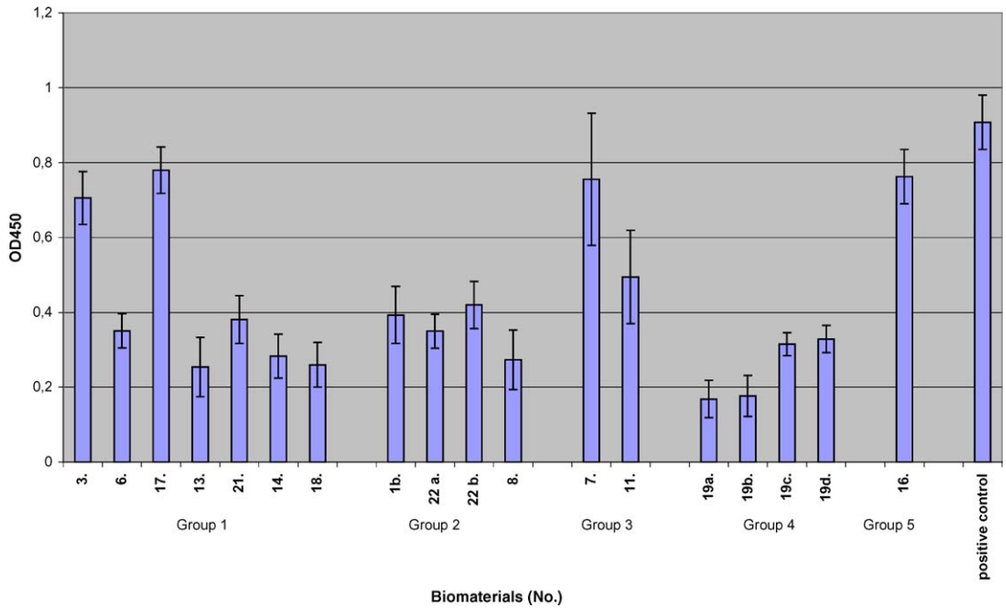
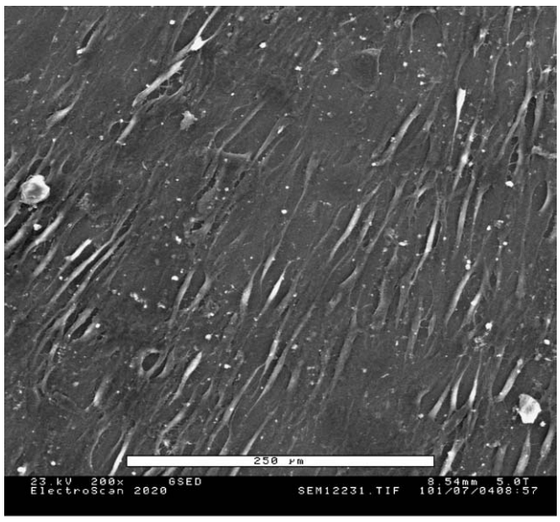


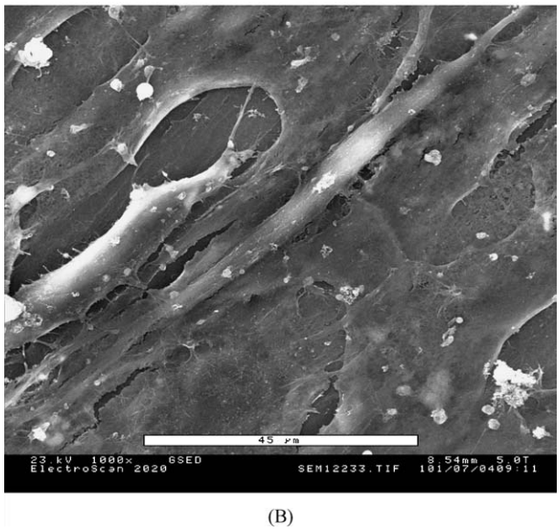
Figure 1 Cell proliferation analysis of human osteoblast-like cells seeded onto 16 different biomaterials divided into five groups. Group 1: Collagen-based membranes, group 2: Tricalciumphosphate-based membranes, group 3: Hyaluronic acid-based membranes, group 4: Anorganic bovine bone membrane, group 5: Anorganic silicone-based membrane. As positive control cells without any biomaterial were used. Positive control: human osteoblast-like cells grown in the culture dish without any biomaterial.

biomaterials were studied by the EZ4U –EASY FOR YOU test. The human osteoblast-like cells seeded onto the biomaterials were allowed to proliferate over a culture period of one week. The average of the measured absorbance and the standard deviation are shown in Fig. 1. The values of the standard deviation for the data series are between 0.045 and 0.17. There were obvious differences in the proliferation rate of the osteoblast-like cells on the different biomaterials tested. The determined high proliferation values of the biomaterials No. 3, No. 7, No. 16 and No. 17 in comparison with the positive control (growth of human osteoblast-like cells in the cell culture dish without any biomaterial) suggest that these membranes are optimal tools for transplantation as well as for the later bone regeneration. Thus, they are also optimal tools for tissue engineered growth of human bone. All four biomaterials with the best proliferation rate and with the most vital cells were not of identical origin: two collagen-based membranes, one hyaluronic acid-based membrane and one anorganic silicone-based membrane. Whereas the biomaterials No. 19a and No. 19b, anorganic bovine bone membranes, showed the worst proliferation rate and barely vital cells (Fig. 1). All other biomaterials examined in this study showed an ordinary proliferation rate and it could be shown that the treatment of biomaterial No. 19b in the perfusion chamber (No. 19c) as well as with additional use of vacuum (No. 19d) enhanced the proliferation rate (Fig. 1).

The surface of all biomaterials with seeded human osteoblast-like cells and without cells was studied via Environmental Scanning Electron Microscope (ESEM). These ESEM examinations revealed attachment and morphology of osteoblasts. Fig. 2 shows the positive control of human osteoblast-like cells grown on glass slides. A high cell density of intact osteoblasts and multiple cell layers are visible. The biomaterials seeded with osteoblast-like cells showed different results: The best results were observed with the biomaterials No. 3, No. 7, No. 16 and No. 17 whereas the worsest growth was observed with the biomaterials No. 19a and No.19b. Ordinary results were achieved with the biomaterials No. 1, No. 6, No. 8, No. 11, No. 13, No. 14, No. 18, No. 19c, No. 19d, No. 21, No. 22a and No. 22b. The biomaterials No. 3, No. 16 and No. 17 without cells are shown in Fig. 3. All three membranes shows different structures: Membrane No. 3 shows a rough surface with an extensive pore mesh, membrane No. 16 appears thread-like and membrane No. 17 shows a smooth surface with lots of pores. Fig. 4 shows the human osteoblast-like cells seeded onto these three biomaterials. The cells were well spread and flattened on the surface of these tested biomaterials. Additionally a very high cell density was observed. The osteoblasts on biomaterial No. 7 showed nearly the same growth behaviour as the osteoblasts grown on glass slides (Fig. 2; biomaterial No. 7 not shown). Fig. 5 shows the biomaterial No. 19a without cells (Fig. 5A) in direct comparison with the seeded cells on this biomaterial (Fig. 5B). The biomaterial No. 19a has a very smooth structure with less and small pores (Fig. 5A). Only few cells were seen to adhere to the membrane No. 19a; the most cells were rounded and seemed to be not viable anymore (Fig. 5B). Similar results were achieved with biomaterial No. 19b (Fig. 6A without cells, Fig. 6B with cells) whereas the treatment of biomaterial No. 19 b in the perfusion chamber for 48 h (Fig. 6C) and the treatment of No. 19b in the perfusion chamber with additional use of 5 min vacuum (Fig. 6d) showed much more vital cells. The cells spread well on the surface of the biomaterial. Fig. 7 (without cells) and Fig. 8 (with cells) show membrane No. 18 and membrane No. 22a as two



(A)



(B)

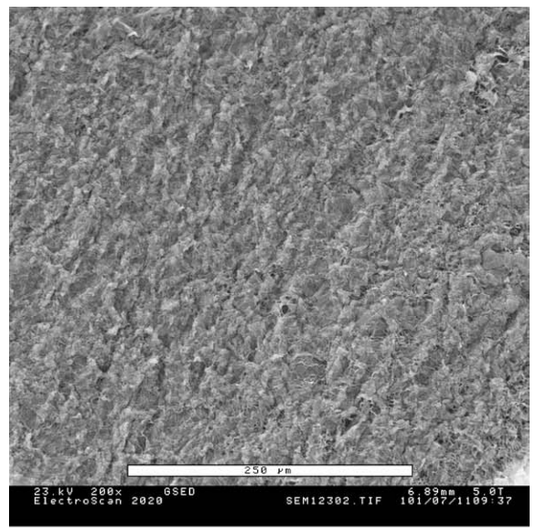
Figure 2 Environmental scanning electron microscopy study of human osteoblast-like cells grown on glass slides: (A) Magnification 200× and (B) 1000×.

examples with an ordinary result in spreading and adherence of the cells on the biomaterials. In Fig. 7 it could be observed that the biomaterial No. 18 (Fig. 7A) has a rough surface with less pores whereas biomaterial No. 22a (Fig. 7B) is grained with lots of pores. The cells on both biomaterials (Fig. 8A and Fig. 8B) were less spread on the surface compared with Fig. 4 but showed anyway a good cell density. Some round cells and less adhered cells were seen.

In all tested biomaterials no differences were visible between the patient material used (data not shown).

4. Discussion

In recent years osseous defects were treated with autologous bone, allogeneic bone or biomaterials such as hydroxyapatite [1]. The use of autologous bone was established as the golden standard although significant disadvantages were observed such as the availability of the bone graft and the morbidity following surgical



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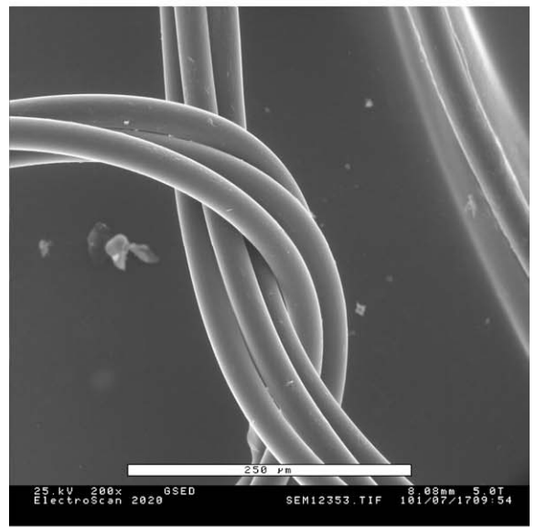

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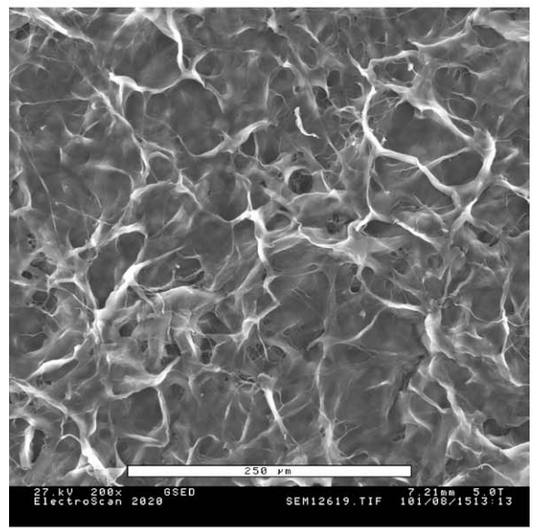
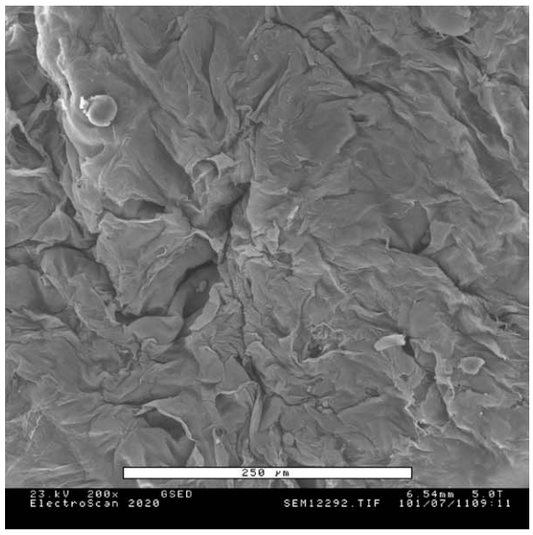


(C)

Figure 3 Environmental scanning electron microscopy study of three biomaterials without human osteoblast-like cells: (A) No. 3 (Tissue Vlies®), (B) No. 16 (Biobrane®) and (C) No. 17 (Biomend™). Magnification 200×.



(A)

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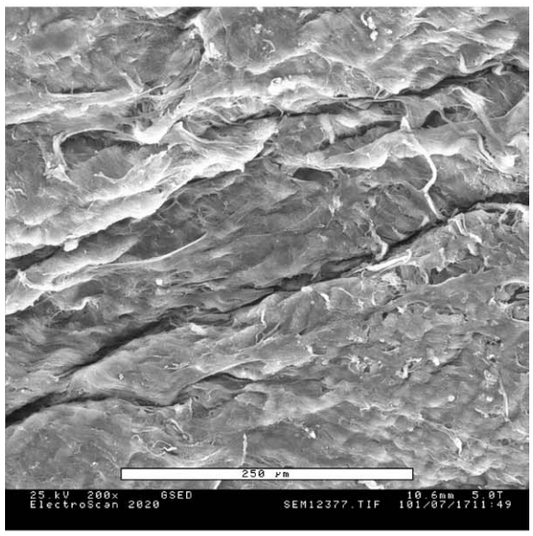
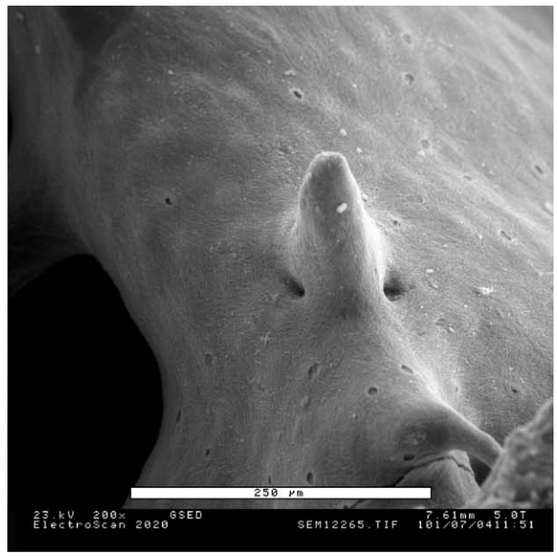
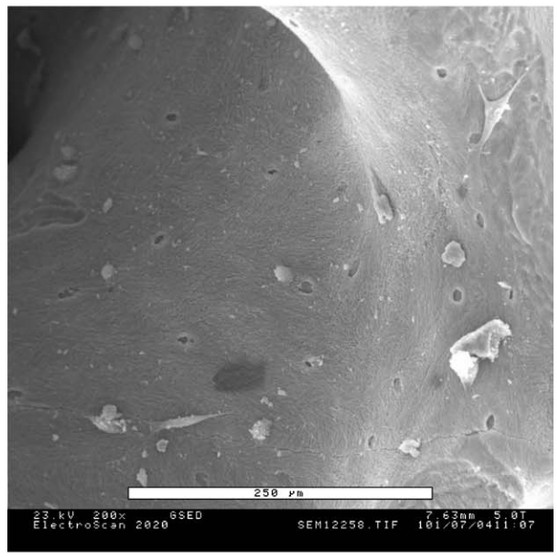


(C)

Figure 4 Environmental scanning electron microscopy study of human osteoblast-like cells cultivated on three different biomaterials: (A) No. 3 (Tissue Vlies®), (B) No. 16 (Biobrane®) and (C) No. 17 (Biomend™). Magnification 200×.



(A)



(B)

Figure 5 Environmental scanning electron microscopy study of biomaterial No. 19a (Bio-Oss®) without human osteoblast-like cells (A) and with human osteoblast-like cells (B). Magnification 200×.

procedures. The use of allogeneic bone, implies also a risk factor considering the transmission of infectious diseases. The application of biomaterials is mostly not satisfied because of the low osseous integration [1]. Consequentely, the search for an ideal biomaterial, which is biocompatible, not cytotoxic and onto which human osteoblast-like cells show a high cell proliferation and cell density are of main interest. The search of such biomaterial/cell constructs with an optimal osseous integration has priority in the field of tissue engineering.

In this study the seeded cells could proved as osteoblasts by the determination of osteocalcin, the determination of collagen type I and by the alkaline phosphatase activity [17]. Osteocalcin is an extracellular non collagenous matrix protein, produced exclusively by osteoblasts. The positive alkaline phosphatase

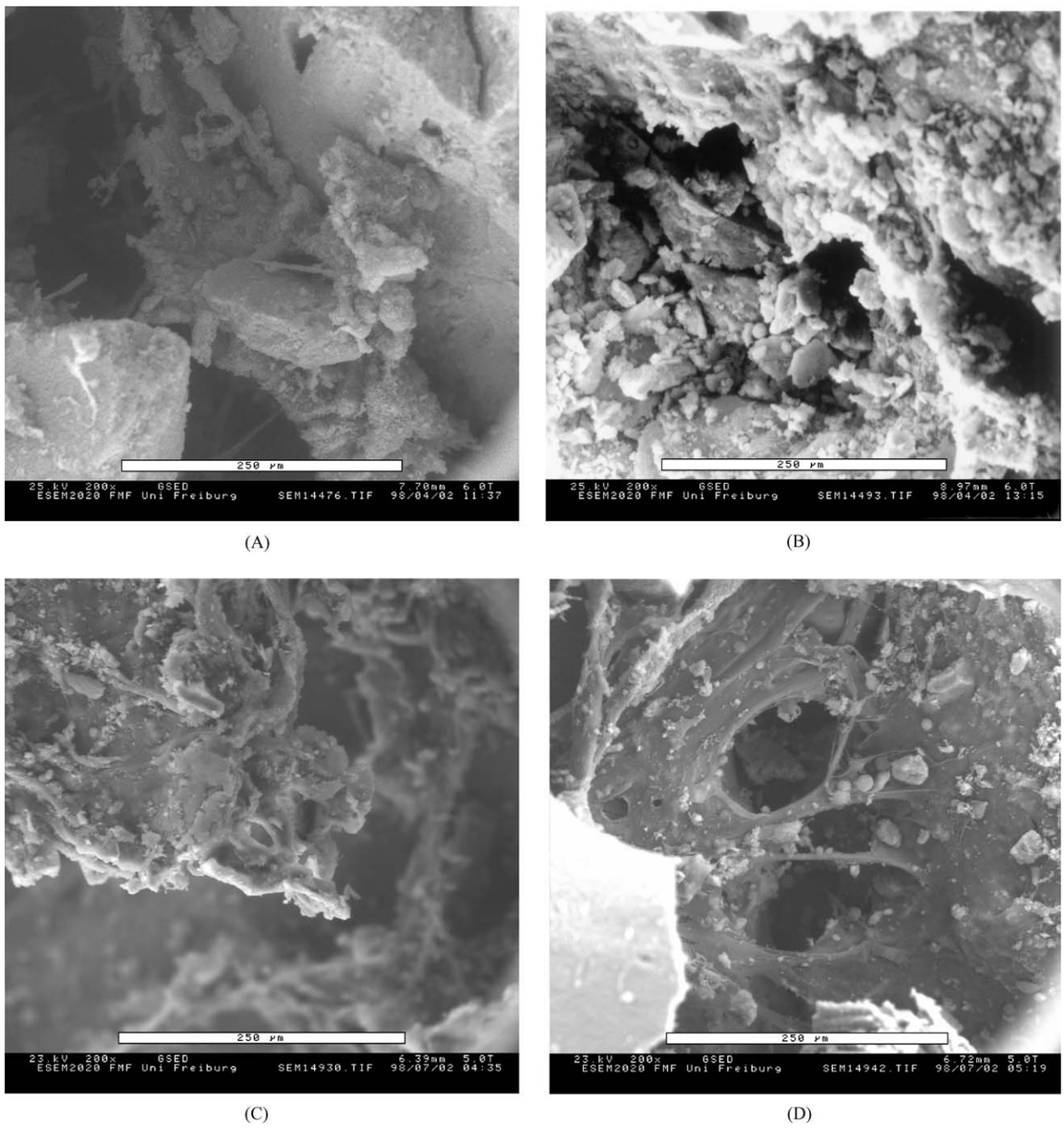
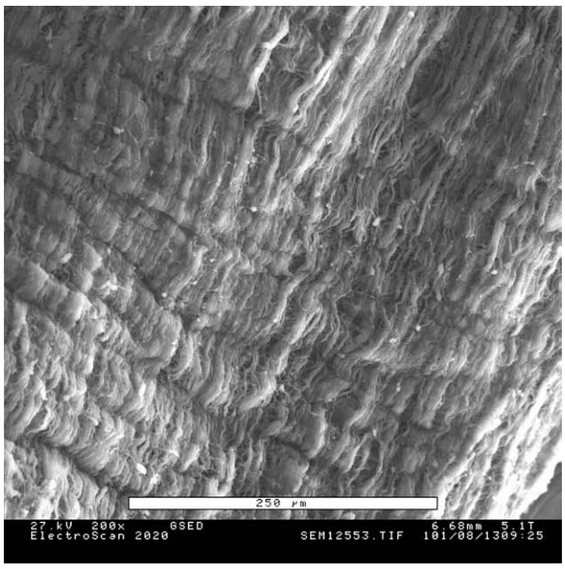


Figure 6 Environmental scanning electron microscopy study of biomaterial No. 19a (Bio-Oss® Collagen) without human osteoblast-like cells (A), with human osteoblast-like cells (B), after treatment in the perfusion chamber for 48 h (C) and after treatment in the perfusion chamber for 48 h with additional use of vacuum for 5 min (D). Magnification 200×.

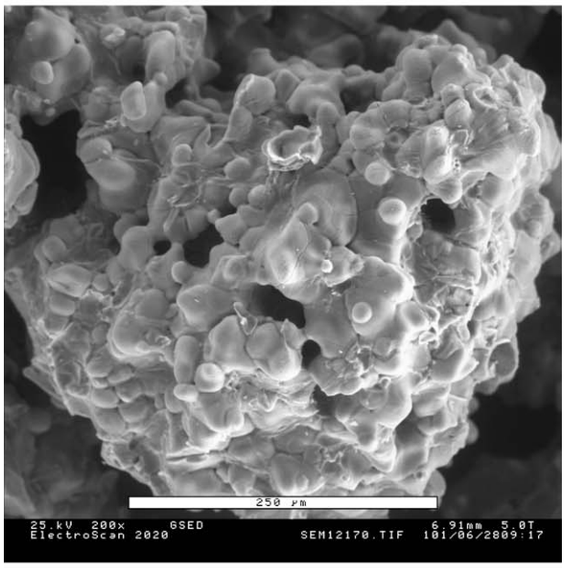
staining was reported in many studies as an indicator for osteoblasts, for the formation of new bone and as a marker for cell differentiation in cultures [18–20]. In our study the high alkaline phosphatase activity indicate that the membranes support the proliferation as well as the differentiation. Such findings were also reported by Lee *et al.* [8]: They used chitosan/tricalcium phosphate sponges as tissue engineered scaffolds as a matrix for fetal rat calvarial osteoblasts and they observed a high alkaline phosphatase activity.

The osteoblast-like cells seeded on the biomaterials No. 3, No. 7, No. 16 and No. 17 showed the best vitality and proliferation rate using the EASY FOR YOU test (Fig. 1). The ESEM analysis confirmed these results: The osteoblasts seeded onto these biomaterials were well attached to the sponge matrices and showed multiple cell layers (Fig. 4). These materials are all from different groups: Materials No. 3 and No. 17 are collagen-based membranes whereas material No. 7 is a hyaluronic acid-based membrane and No. 16 an anorganic-based silicone membrane. Consequentely, no favourable group of biomaterials is noticeable. Moreover, the results indicate that all these biomaterials as a part of bone constructs are the best tools for engineering new bone tissue.

In contrast, the biomaterials No. 19a and No. 19b, anorganic bovine bone membrane (Bio-Oss® and Bio-Oss® Collagen), showed the lowest proliferation rate in our study (Fig. 1). Nearly no vital cells were visible on the surface of the biomaterial (Figs. 5B and

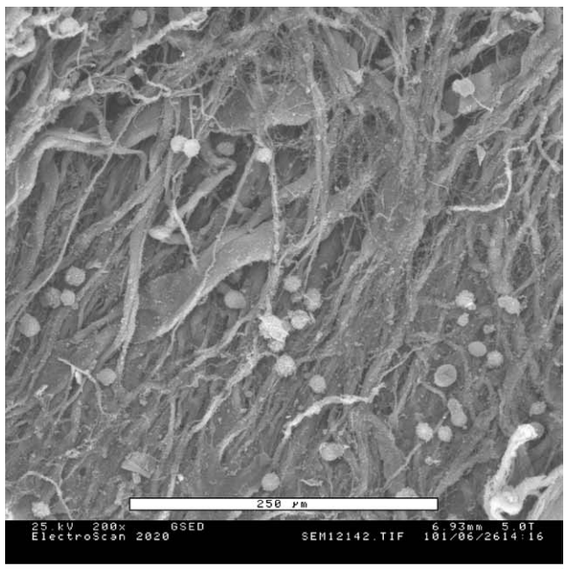


(A)

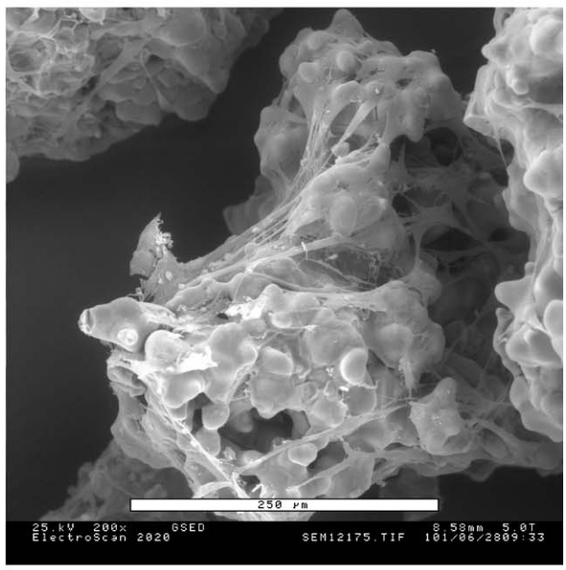


(B)

Figure 7 Environmental scanning electron microscopy study of two biomaterials without human osteoblast-like cells: (A) No. 18 (Bio-Gide) and (B) No. 22a (Bio Resorb). Magnification 200×.



(A)



(B)

Figure 8 Environmental scanning electron microscopy study of human osteoblast-like cells cultivated on two different biomaterials: (A) No. 18 (Bio-Gide) and (B) No. 22a (Bio Resorb). Magnification 200×.

6B). This is explainable because of the smooth surface of this biomaterial which affects the adherence of the cells: The cells drift on the ground of the culture plates and are not capable to fix on the biomaterial. For this anorganic bovine bone membrane different results in clinical conditions were reported: This biomaterial has been shown to be highly biocompatible with oral hard tissues and to fullfill the criteria of an osteoconductive material [9, 21–24]. Piattelli *et al.* [9] used this biomaterial for sinus augmentation procedures and was able to show that it was very useful as a bone substitute in maxillary sinus augmentation leading to appropriate osseointegration. Acil *et al.* [25] studied the growth of human osteoblast-like cells on this highly porous natural bone mineral. This study demonstrated multiple cell layers by scanning electron microscopy on the surface of the matrix. Moreover, transmission electron microscopy examinations showed also cells in the internal spaces. These findings are completely in contradiction with our results: no viable cell was visible anymore, nearly no proliferation rate could be observed in our present study (Figs. 1 and 5B). One reason for these different results could be that Acil *et al.* [25] used trabecular bone whereas in our study cortico-lamellar bone of the maxilla was used. These cells could develop a different growth behaviour. Another reason could be the different culture conditions (e.g. seeding density, cultivation duration) of both studies. To compare directly all 16 biomaterials we used a constant seeding density and a constant cultivation duration. In our recent study [13] we already examined the seeding and cultivation procedure in order to optimize the attachment

and growth of human osteoblasts on three various cell carriers (a native bovine collagen membrane, a native equine collagen membrane and a native calf collagen membrane). The results of this recent study showed that the time interval between seeding osteoblasts and adding culture medium as well as the seeding concentration significantly affect the osteoblast proliferation. The best proliferation rate and attachment was achieved using a seeding density of 10^5 cells/ml and with 2 h adding the culture medium after seeding the cells. For scanning electron microscopy study we used a culture period of one week. These results were the base for this study. In contrast, Acil *et al.* [25] did this scanning electron microscopy examination after six weeks. If we used such a long cultivation duration the osteoblasts would not be viable anymore especially in the case of the studied biomaterials which have a very good proliferation rate (No. 16, No. 7, No. 17 and No. 3). No direct comparison between the biomaterials would be possible anymore. Another reason for this short cultivation duration is the requirement that a fast subsequent grafting of such biomaterial/cell constructs is possible, especially in emergencies.

Interestingly, the treatment of biomaterial No. 19b in the perfusion chamber for 48 h in cell culture medium (No. 19c) and the additional use of vacuum for 5 min (No. 19d) enhanced the proliferation rate and the cell growth (Figs. 1, 6C and D). The reasons for this growth improvement could be that with the help of the perfusion chamber the medium is able to invade better into the biomaterial. An additional vacuum treatment for 5 min leads to the removement of the oxygen inside the biomaterial. Consequentely, this offers better conditions for the growth and invasion of the osteoblast like cells.

Biomaterial No. 18, a native pig collagen membrane (BioGide), is one of the materials onto the osteoblasts show an ordinary proliferation rate and a good cell density on the surface although some round cells were observed. Alpar *et al.* [6] compared the growth of human osteoblast-like cells (SAOS-2) incubated with this resorbable collagen membrane with polylactic acid membrane by scanning electron microscopy. They could show that the collagen membrane was dense populated with the osteoblasts in contrast to the polylactic acid membrane. This densely population of the osteoblasts on the collagen membrane is in accordance with our study.

5. Conclusion

Human osteoblast-like cells cultivated on 16 biomaterials showed in four cases a very good proliferation rate, vitality and a high density. The findings also showed the possibility of subsequent grafting of such constructs and the importance in tissue engineering compared with the use of autologous bone. Moreover, the results offer the basis for further studies such as transplatation assays of such biomaterial/osteoblast constructs in humans. Especially examinations about the degradation and bone regeneration of such cell/matrix structures as well as detailed knowledge of the interaction between cells and materials would be of high interest.

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