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Comparative in vitro study of the proliferation and growth of ovine osteoblast-like cells on various alloplastic biomaterials manufactured for augmentation and reconstruction of tissue or bone defects

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Abstract In this in vitro study ovine osteoblast-like cells were cultured on seven different alloplastic biomaterials used for augmentation and for reconstruction of bone defects in dental and craniomaxillofacial surgery. The aim of this study was to examine the growth behaviour (viability, cell density and morphology) of ovine osteoblast-like cells on the investigated biomaterials to get knowledge which biomaterial is qualified to act as a cell carrier system in further in vivo experiments. The biomaterials were either synthetically manufactured or of natural origin. As synthetically manufactured biomaterials Ethisorb®, MakroSorb®, Palacos®R, and PDS® film were used. As biomaterials of natural origin Beriplast®P, Bio-Oss® and Titanmesh were investigated. The cell proliferation and cell colonization were analyzed by a proliferation assay and scanning electron microscopy. Osteoblast-like cells proliferated and attached on all biomaterials, except on Beriplast®. On Ethisorb® the highest cell proliferation rate was measured followed by Palacos®R. Both biomaterials offer suitable growth and proliferation conditions for ovine osteoblast-like cells. The proliferation rates of Bio-Oss®, MakroSorb®, PDS®-film and Titanmesh were low and SEM examinations of these materials showed less spread osteoblast-like cells. The results showed that ovine osteoblast-like cells appear to be sensitive to substrate composition and topography. This in vitro study provides the basis for further in vivo studies using the sheep model to examine the biocompatibility and the long-term interaction between the test material and tissue (bone regeneration).

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1 Introduction

Over the past decades the use of autologous cortical bone and cancellous bone grafts have been widely accepted as the most compatible material for clinical surgery. Bone grafts are commonly required in orthopedic, oral, and maxillofacial surgery for various indications [1–4]. In cases of skeletal deficiencies and after surgical procedures i.e. augmentations for dental implants, and reconstruction of bone lost during tumor removal or trauma bony grafts are required [3, 5, 6].

Presently surgeons treat hard tissue defects with autogenous bone, the gold standard for clinical surgery [7–14]. However, autogenous grafts have their associated problems. Morbidity at the donor site and devitalisation of the autogenous transplant are major drawbacks of bone grafts due to resorption processes during healing [4, 15–20]. The disadvantages have encouraged the development of alternative materials, such as allogenous grafts and/or alloplastic biomaterials. The application of allogenous bone material has a potential higher risk of infections and is also more expensive [3, 21–23]. The use of alloplastic biomaterials has many advantages i.e. availability and an almost inexhaustible amount of material. But alloplastic biomaterials do not reach good results on their own properties. Recently, they were combined with cellular

components i.e. cells, growth factors or cytokines [6, 24–26]. The composed biomaterials showed significant better regeneration as biomaterials alone [21, 27–30]. The success of this approach is highly dependent on the properties of the material. Biocompatibility, sterilizability and osteoconductivity are the prerequisites [2, 31–34]. Among alloplastic biomaterials there are resorbable and non resorbable materials. They differ in their components and substances, in their surface characteristics as well as in their physical and chemical properties [20, 32–36]. In cell culture studies with osteoblast-like cells the surface characteristics of the material play an important role because osteoblastic cells require a supportive matrix in order to survive [22, 37–42]. The surface allows the attachment of the cells, supports cell growth and the differentiation of cells. The material must also provide an appropriate environment for the proliferation and function of osteoblast-like cells [35, 39, 43]. In addition to commercially available alloplastic biomaterials there are alloplastic materials which were especially created for different kinds of experiments [4, 31, 38, 44].

In this study we investigated seven different commercially available resorbable and non resorbable alloplastic biomaterials. Four materials were of synthetic origin, whereas three materials were of natural origin. They differed in material and surface characteristics. All materials were cultivated with ovine osteoblast-like cells.

The aim of the present in vitro study was to examine the growth behaviour of ovine osteoblast-like cells on different alloplastic materials. Besides cell viability we also monitored the effect of the investigated materials on the density and morphology of ovine osteoblast-like cells to get knowledge which biomaterial offers the best conditions for the growth of osteoblast-like cells. We determined whether the various biomaterials are qualified to act as a cell carrier system for subsequent use in animal (sheep) experiments or not. Further in vivo studies using the sheep model are necessary to examine the biocompatibility and the long-term interaction between the test material and tissue. Additionally, the in vivo experiments determine whether these biomaterials are composed of substances which will enhance the process of bone reconstruction and remodelling.

2 Materials and methods

2.1 Biomaterials

Seven commercially available biomaterials were used for cultivation experiments with ovine osteoblast-like cells. The investigated materials which were either synthetically manufactured or of natural origin are used as tissue/bone substitutes, for augmentation and for reconstruction of lost hard tissue in maxillofacial surgery or bone defects.

As synthetically manufactured biomaterials Ethisorb® Dura Patch (Johnson & Johnson Intl., Ethicon GmbH & Co KG, Norderstedt, Germany; resorbable/biodegradable), MakroSorb®FX (Macro Pore, San Diego, CA, USA; poly(L/DL-lactide); resorbable/biodegradable), Palacos®R (Merck Biomaterial GmbH, Darmstadt, Germany; acrylic bone cement with high viscosity, nonresorbable) and PDS®-film (Johnson & Johnson Intl., Ethicon GmbH & Co KG, Norderstedt, Germany; poly-p-dioxanon homopolymer, resorbable/biodegradable) were used. As biomaterials of natural origin Beriplast®P (Aventis Behring GmbH, Marburg, Germany; fibrin glue, resorbable/biodegradable), Bio-Oss® (Geistlich Biomaterials, Schlieren, Switzerland; natural bone mineral of bovine origin, nonresorbable) and Titanmesh (Synthes AG Chur, Oberdorf, Switzerland; nonresorbable) were investigated.

2.2 Primary explant culture

Ovine osteoblast-like cells were isolated from bone biopsies taken from the mandible of seven sheep skulls.

For the attempt on two different culture media we cultivated ovine osteoblast-like cells from four sheep skulls. The isolation and culturing methods were the same during both experiments.

The biopsies were dissected, cut into small fragments (1 mm^3) and extensively washed in phosphate-buffered saline (PBS). The fragments were seeded as explants on culture flasks (25 cm^2, Falcon, Heidelberg, Germany) using BGJb medium (Gibco Laboratories Life Technologies, Inc, Grand Island, NY, USA) and Opti-minimal essential medium (Opti-MEM-I, Gibco Laboratories Life Technologies, Inc, Grand Island, NY, USA), respectively. The cell cultures were kept in a humidified atmosphere of 5% CO_2 at 37 °C (Heraeus, Hanau, Germany). The medium was replaced twice a week. After 8 days of cultivation, cells migrated out of the bone fragments and settled on the floor of the culture flasks.

2.3 Subcultivation

For subcultivation the confluent osteoblast cultures were enzymatically detached from the culture flasks by trypsin treatment. In brief, after removing the medium, the cells were incubated with 0.5% trypsin (Biochrom AG, Berlin, Germany) in Ca^{2++} and Mg^{2++} free 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 (Greiner, bio-one GmbH, Frickenhausen, Germany),

centrifuged (Biofuge Strato, Heraeus, Hanau, Germany; 1120*g*, 12 min, 20 °C) and resuspended in 1 mL culture medium (first passage). The cells were transferred in a 75 cm^2 culture flask (Greiner, bio-one GmbH, Frickenhausen, Germany), and 30 mL culture medium were added. An incubation in humidified atmosphere followed.

The osteoblast-like cells which migrated onto the floor of the culture flasks formed a confluent layer after 5–6 weeks. These cells were enzymatically detached again with 0.5% trypsin, centrifuged and resuspended in 1 mL medium (second passage). After a cultivation period of 7 weeks the osteoblast-like cells were used for the attempt on different culture media and for the cell culture experiments on the various biomaterials.

2.4 Attempt on culture media

Until now, no special culture medium for ovine osteoblast-like cells was described. To define the optimal medium for cultivation of ovine osteoblast-like cells we investigated two different culture media, BGJb and Opti-MEM-I. BGJb was developed for embryonic tibiae organ culture, whereas Opti-MEM-I is a widely used culture medium.

Both culture media were supplemented with 10% foetal calf serum (FCS, PAA Laboratories GmbH, Linz, Austria), 4% Hepes (Gibco Laboratories Life Technologies, Inc, Grand Island, NY, USA), and 1% penicillin–streptomycin (Biochrom AG, Berlin, Germany).

From bony biopsies of four sheep skulls ovine osteoblast-like cells were isolated and cultured as described above. Cells corresponding to one sheep were cultured in both growth media at the same culture conditions and under the same treatment. After a total of 12 weeks 2×10^4 cells of the second passage were seeded in triplicate into wells of a 96 well microplate (Corning Inc, NY, USA) and filled up with BGJb and Opti-MEM-I, respectively. The cultures were kept in humidified atmosphere of 5% CO_2 at 37 °C. The medium was changed every second day with fresh aliquots. To compare the two different media with each other a cell proliferation assay was performed after a total of 7 days. The medium that reached the highest cell proliferation rate was used for cell seeding onto various biomaterials.

2.5 Assay for osteocalcin

For the detection and quantification of osteocalcin in the cell culture supernatant of ovine osteoblast-like cells, the Metra™ Osteocalcin immunoassay (Quidel Corporation, San Diego, CA, USA) was performed before seeding the cells onto the biomaterials. The test is a competitive immunoassay which is highly specific for intact osteocalcin and quantitatively measures intact osteocalcin in serum or supernatant. The test was performed according to the manufacturer's instructions. In brief, 25 µL standards, the curve controls, and the cell culture supernatants were added to each well of the coated strips in the 96 well microplate. 125 µL anti-osteocalcin antibodies were added to each well and incubated for 2 h at room temperature (20–25 °C). All wells were washed three times with 1× wash buffer and then 150 µL reconstituted enzyme conjugate were added to each well. At room temperature the 96 well microplate was incubated for 60 min. Once again the wells were washed three times with 1× wash buffer followed by adding 150 µL working substrate solution to each well. After an incubation time of 35–40 min at room temperature the reaction was stopped by 50 µL stop solution and the optical density (OD) was measured at 405 nm.

Osteocalcin is exclusively synthesized by osteoblasts and is believed to prevent premature mineralization of newly formed, but yet disorganized bone matrix.

2.6 Van Kossa silver nitrate staining

About 6 weeks after cell seeding ovine osteoblast-like cells were examined under light microscope by van Kossa staining. The cells were cultured in six wells of a 24 well culture plate at a density of 1×10^6 cells/well. They were washed with Tris-buffered saline (TBS) in order to remove all free phosphorus, and rinsed with water. Finally, an $AgNO_3$ solution (5%; w/v) was poured into each well, and then the cultures were fixed with $Na_2S_2O_3$.

The method of van Kossa makes evident the presence of insoluble calcium phosphate salts in the extracellular spaces, which is specific for bone cells.

2.7 Cell seeding onto the biomaterials

The sterile biomaterials (10×10 mm^2 in size) were placed in double into two wells of 24 well culture plates (Costar, NY, USA) for subsequent cell seeding, followed by scanning electronic microscopic examinations. Additionally, one well with the biomaterial only was used as control. All biomaterials were used without pre-wetting, except of Bio-Oss® according to the manufacturers instructions. Before cell seeding, this material was incubated in culture medium for 24 h. Afterwards the medium was exhausted and new medium was added for 1 h before the cells were seeded onto the biomaterial.

In parallel for the proliferation assay three smaller samples (4×5 mm^2) of each material were placed in triplicate in a 96 well microplate and seeded with cells.

One additional well contained the medium blank without cells and one well the biomaterial only.

Cells were seeded after trypsination as described above. Cell number and viability were determined by cell counts using a haemocytometer and trypan blue exclusion. 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). About 100 μL cell suspension at a concentration of 1×10^6 cells/mL were seeded onto each 10×10 mm^2 biomaterial with a sterile syringe. Into each well of the 96 well microplates, 20 μL of the same cell suspension were inoculated onto the smaller pieces of material. Cells were allowed to adhere on the material for 2 h at 37 °C in 5% CO_2 atmosphere, before culture medium to a total volume of 1 mL was added to each well of the 24 well culture plates, and to each well of the 96 well microplates to a total volume of 200 μL.

One 24 well plate consisted of cells without any biomaterial. As negative control the polystyrene floor of the well plate was used.

The cell cultures were kept in humidified atmosphere. Every second day the culture medium was changed. After an incubation period of 7 days, scanning electron microscopic examinations and proliferation assays followed. Additionally, supernatants from all cultures were collected for evaluation of cytotoxicity and cell proliferation.

2.8 Cell proliferation analysis

To evaluate cell proliferation and viability the EZ4U—EASY FOR YOU test (Biomedica GmbH, Wien, Austria) was performed. The test is a non-toxic, non-radioactive, reliable non-isotopic cell proliferation assay based on tetrazolium salts which yield soluble reduction products. The method is based on the finding that living cells are capable to reduce slightly yellow or uncoloured tetrazolium salts into intense red coloured formazan derivatives. The formazan derivatives are excreted into the culture medium and the absorbance can be measured with a microplate-reader. The amount of coloured formazan derivatives correlates with the amount of living cells in the sample. The proliferation assay was carried out according to the manufacturer's instructions. In brief, 20 μL of EZ4U dye solution were added to 200 μL culture medium in each well. The 96 well microplates were incubated at 37 °C in CO_2 atmosphere for 4 h. The absorbance was measured by a microplate-reader and quantified spectrophotometrically at 492 nm with 620 nm as reference. As control, ovine osteoblast-like cells which were grown without any biomaterial were used. The assay was performed in triplicate with osteoblast-like cells from seven sheep. Results are reported as OD.

2.9 Scanning electron microscopy

Cell colonization, cell morphology and cell attachment analysis of the investigated biomaterials were assessed by scanning electron microscopy (SEM) after an incubation period of seven days onto the 10×10 mm^2 samples.

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 ethanol series (30, 50, 70, 80, 90% once for 1 h, 99.8% twice for 1 h), followed by critical-point drying with liquid CO_2 in a CO_2 atmosphere with 90 bar at 42 °C (CPD 030, Bal-Tec, Wallruf, Germany). Finally, the samples were mounted on aluminium stubs for SEM analysis, grounded with a coalfilm and silver glue, and sputter coated with a thin film with gold–palladium (Au–Pd, Plano, Germany). The samples were examined at 13–15 kV, 70 mA (LEO 435 VP, Zeiss, Kochern, Germany). Images were recorded digitally.

2.10 Statistical analysis

Statistical analysis was performed by ANOVA and Tukey test using the SPSS 15.0 software package. The p values of ≤ 0.05 indicate significantly differences, whereas p values of ≤ 0.001 indicate high significantly differences.

3 Results

3.1 Attempt on culture media

The vitality and the proliferation capacity of ovine osteoblast-like cells from four different sheep seeded and cultured with two different culture media were studied by the EZ4U—EASY FOR YOU test. The average of the measured absorbance is shown in Fig. 1. The values of the standard deviation were between 0.088 and 0.064 (see Fig. 1, error bars). Ovine osteoblast-like cells cultured with BGJb showed in each test significantly more vital cells than with Opti-MEM-I.

3.2 Cell proliferation and vitality

The cell vitality and proliferation capacity of ovine osteoblast-like cells cultivated onto seven biomaterials were measured by the EZ4U test. The highest proliferation rate was measured in the cultures used as control (ovine osteoblast-like cells on the polystyrene floor of the culture wells without any biomaterial) whereas the proliferation rates of the cells cultivated on the investigated biomaterials

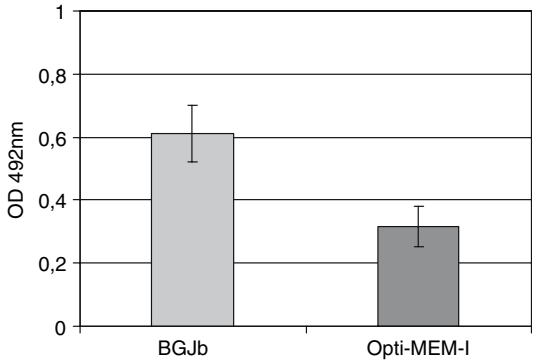

Fig. 1 Cell proliferation analysis of ovine osteoblast-like cells cultured in two different media (BGJb and Opti-MEM I, respectively). Error bars represent standard deviations

were significantly lower ($p < 0.001$). The average of the measured absorbence ($OD_{492\ nm}$) is shown in Fig. 2.

The osteoblast-like cells cultivated on the investigated biomaterials showed significant differences in the proliferation rates. The highest proliferation rate was measured on Ethisorb® Dura Patch ($OD_{492\ nm} = 0.204$) followed by Palacos®R ($OD_{492\ nm} = 0.088$). In each test Ethisorb® Dura Patch and Palacos®R showed significantly more vital cells than the other biomaterials. Ethisorb® Dura Patch showed a significantly higher proliferation rate compared to all other investigated biomaterials ($p \leq 0.001$). Palacos®R showed a significantly higher proliferation rate compared to Bio-Oss® and Beriplast®P ($p \leq 0.05$) and a less significantly higher proliferation rate compared to MakroSorb®FX ($p = 0.063$) and Titanmesh ($p = 0.07$).

On Bio-Oss®, MakroSorb®FX, PDS®-film and Titanmesh the test measured a very low amount of vital cells. In detail, the average of the measured absorbence on Bio-Oss® was 0.006, on MakroSorb®FX 0.006, on PDS®-film 0.019, and on Titanmesh 0.008. The lowest proliferation rate was measured on Beriplast®P. Just one of nine values on this material could be measured and was positive ($OD_{492\ nm}$ 0.003; in average 0.000).

3.3 Scanning electron microscopy

The morphology, the growth and the shape of the cells on the surfaces of all biomaterials were examined by scanning electron microscopy. All biomaterials had different structures and surfaces. The four synthetically manufactured biomaterials are shown in the Fig. 3A–D without cells as control and in Fig. 4A–D seeded with ovine osteoblast-like cells. The three materials of natural origin are shown in Fig. 5A–C without any cells as control and in Fig. 6A–C with ovine osteoblast-like cells seeded on the surface of the materials.

The SEM revealed that osteoblasts adhered severely to the biomaterial surfaces with considerable differences between the various biomaterials. Ovine osteoblast-like cells differed in their phenotype, their morphology, their size and their density onto the different materials.

Onto Ethisorb® Dura Patch the SEM examination revealed that the cells had attached, multiplied, and covered almost the whole material surface (Fig. 4A). In the presence of Ethisorb® Dura Patch confluent ovine osteoblast-like cell sheets with a very high cell density were observed. The cells spread well on the surface, appeared to be flattened and oriented in a way following the original structure of the material.

In contrast, the cells cultivated on MakroSorb®FX seemed almost detached from the substrate (Fig. 4B). Only a few cells adhered to the smooth surface of the material and many round, not vital cells were observed.

Onto Palacos®R the ovine osteoblast-like cells were well spread, and extended from the surface into the rough interior of the material (Fig. 4C).

The cells grown on PDS®-film grew sporadically on the surface of the material (Fig. 4D). In the presence of the PDS®-film the ovine osteoblast-like cells appeared in a long, thin, cellular extensions.

The worst results were observed onto Beriplast®P (Fig. 6A). Ovine osteoblast-like cells onto Beriplast®P were round and seemed not to be viable anymore.

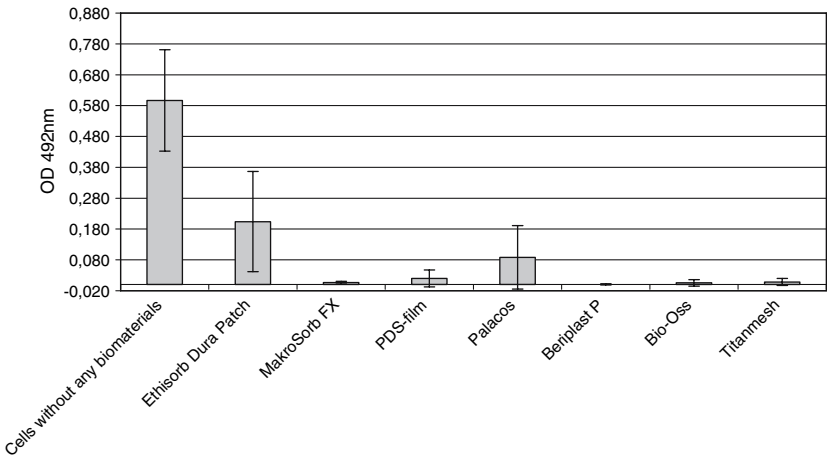

Fig. 2 Cell proliferation analysis of ovine osteoblast-like cells seeded onto seven different biomaterials. The average of the measured absorbencies is shown. Error bars represent standard deviations

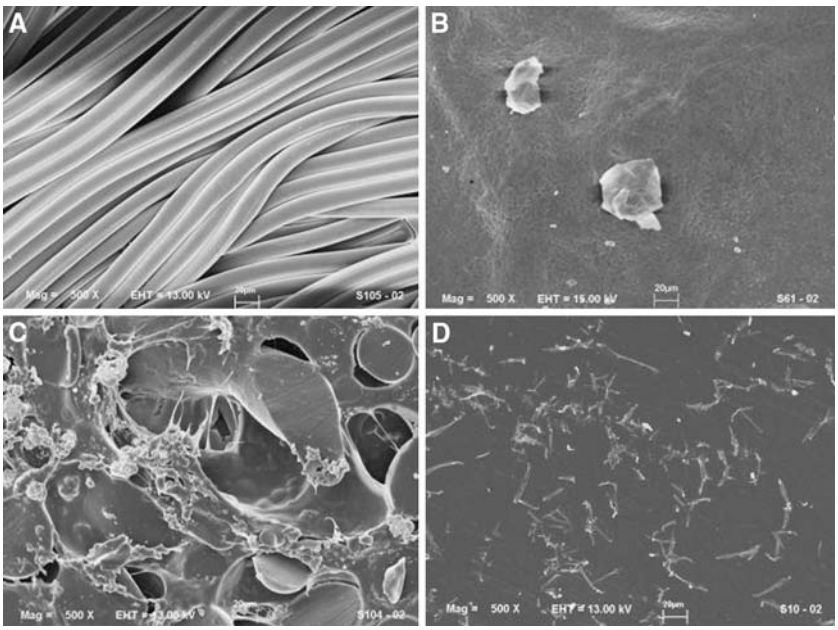

Fig. 3 Scanning electron micrographs of synthetically manufactured biomaterials without any cell cultivation. Ethisorb® Dura Patch (**A**), MakroSorb®FX (**B**), Palacos®R (**C**) and PDS®-film (**D**). Magnification 500×

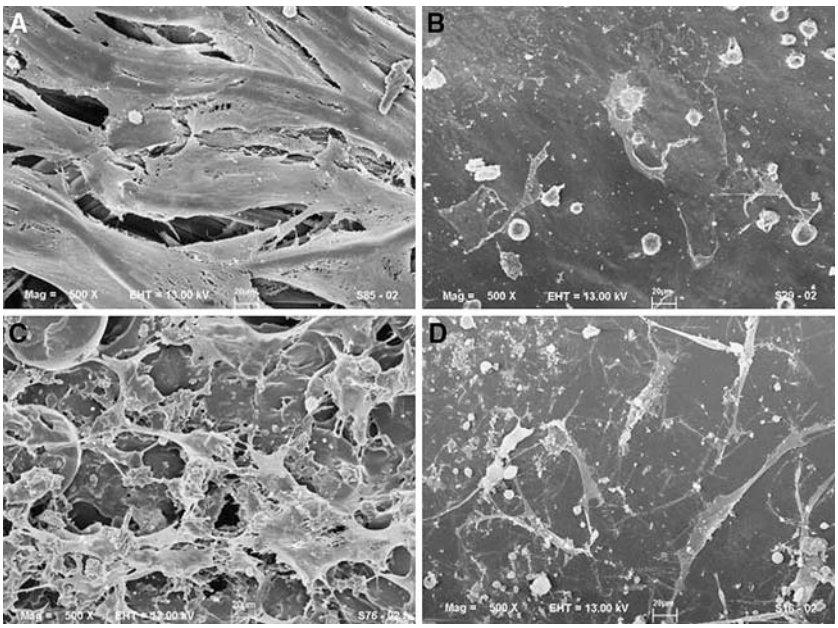

Fig. 4 Scanning electron micrographs of synthetically manufactured biomaterials after cultivation for seven days with ovine osteoblast-like cells. Ethisorb® Dura Patch (**A**), MakroSorb®FX (**B**), Palacos®R (**C**) and PDS®-film (**D**). Magnification 500×

The SEM examination showed that on Bio-Oss® osteoblast-like cells attached sporadically onto the material and the cells appeared as flattened cells with thin, long extensions (Fig. 6B). Osteoblast-like cells on Bio-Oss® showed the same morphology and growth behaviour compared to Titanmesh (Fig. 6C). On both materials the cells were less spread on the surface and a confluent monolayer was not formed.

The metal surface of Titanmesh was covered with isolated long, thin cells with numerous filamentous extensions.

No differences were observed between the investigated bone material of the different sheep (data not shown).

3.4 Identification and characterization

3.4.1 Osteocalcin determination and van Kossa staining

Cells were defined as osteoblast-like cells by the determination of osteocalcin and by the van Kossa staining.

Osteocalcin is the most abundant noncollagenous protein in the mineralized bone matrix. The osteocalcin amount was estimated using the competitive immunoassay provided by Metra™. The average amount of osteocalcin was 7.240 µg/L.

The positive van Kossa staining was identified by light microscopy and confirmed the results of the osteocalcin

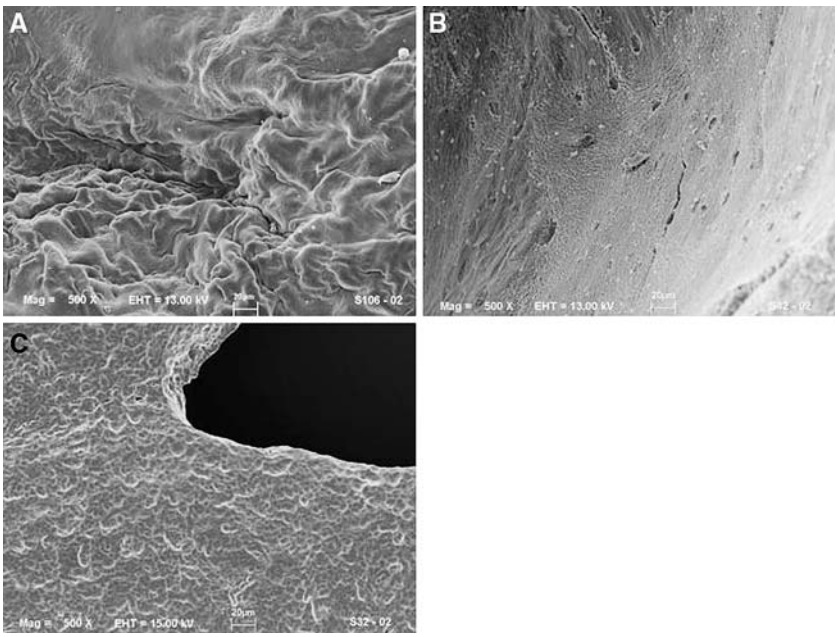

Fig. 5 Scanning electron micrographs of biomaterials of natural origin without any cell cultivation. Bio-Oss® (**A**), Beriplast®P (**B**) and Titanmesh (**C**). Magnification 500×

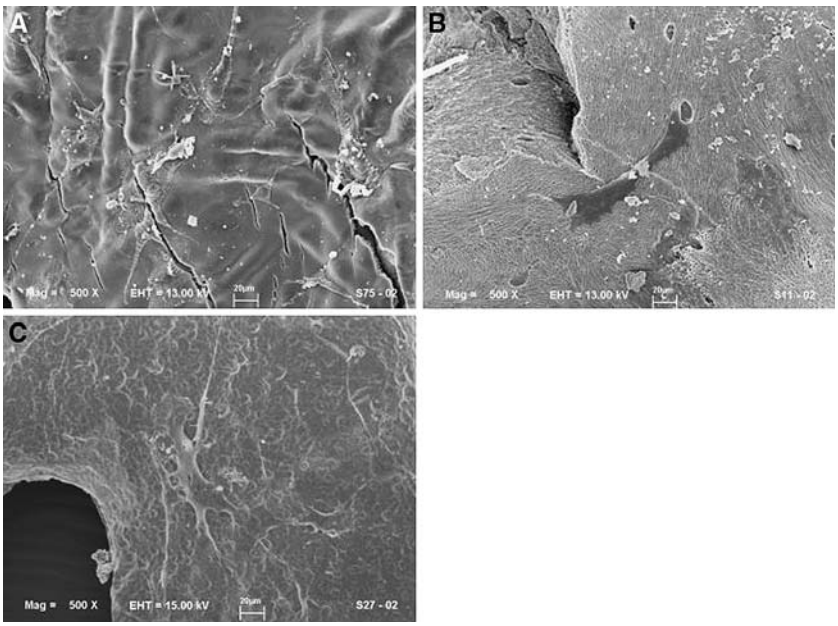

Fig. 6 Scanning electron micrographs of biomaterials of natural origin after cultivation for 7 days with ovine osteoblast-like cells. Bio-Oss® (**A**), Beriplast®P (**B**) and Titanmesh (**C**). Magnification 500×

determination (Fig. 7). The cells were able to differentiate and mineralize in vitro, showing an osteoblastic behaviour.

4 Discussion

The in vitro cell culture model is used for basic science research and for clinical application purposes. This model is used to evaluate the biocompatibility of bone or tissue substitutes and to assess the stability of bone-biomaterial interfaces, and the osseointegration of biomaterials [38, 45]. Furthermore, it is used for subsequent strategies to create ideal graft materials and biologic substitutes [38]. Advantages of this model are to reduce the number of animals required for subsequent in vivo investigations, to gain informations about the cell behaviour onto different substances and to study the properties of biomaterials during cell cultivation [45]. An encouraging new approach of creating biologic substitutes has focused on the use of natural or synthetic materials as cell carrier systems for cell transplantation of osteogenic cells [46].

In the present study we used osteoblast-like cells obtained from the mandible of sheep skulls. Sheep were investigated in this study with regard to later intented in

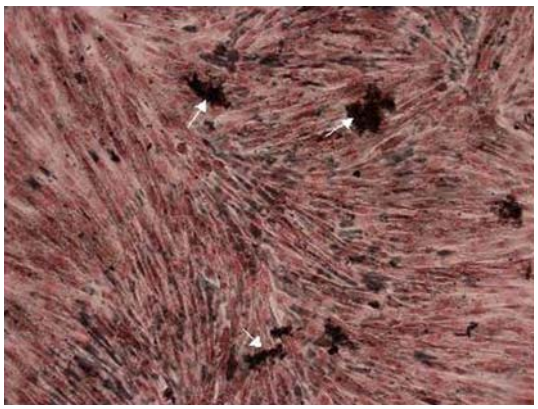

Fig. 7 Light microscopy of ovine osteoblast-like cells after van Kossa silver nitrate staining. Insoluble calcium phosphate salts in the extracellular spaces (➙). Magnification 100×

vivo studies. Spectroscopical analysis showed similarities between sheep and human bones regarding to the micromorphological structure. The mandible was specifically selected because it offers an easily accessible area in later in vivo studies. Sheep are a well accepted model for the examination of bone and have been frequently used to study questions related to bone grafts and substitutes [45].

Two different experiments were investigated and morphological and biochemical analyses were performed. First, an attempt on different cell culture media was carried out to define the effects of different culture media on the proliferation and the growth of ovine osteoblasts as well as to find a growth medium which offers ideal conditions for in vitro culturing of ovine osteoblast-like cells. We isolated and cultured ovine osteoblast-like cells in two different culture media according to the explant cell culture technique. BGJb and Opti-MEM I were used. Both were standard culture media for osteoblast-like cells. A carefully choice of media dependent on the investigated cells were previously recommended [47].

The proliferation rate of the cells in both media was measured by the EZ4U—EASY-FOR-YOU test and results were compared. In BGJb the cell proliferation was significantly higher compared to the proliferation in Opti-MEM-I. The reason why the proliferation in BGJb was more pronounced than in Opti-MEM-I has to be related to the composition of the culture medium. The composition of BGJb caused a better cell growth and a better cell proliferation of ovine osteoblast-like cells. BGJb was therefore used for seeding osteoblast-like cells onto various biomaterials.

We used the second passage of in vitro cultured osteoblast-like cells in agreement with previous studies [48, 49]. For cell seeding onto the biomaterials a cell concentration of 1×10^6 cells/mL according to Uemura et al. [31] was used. After the incubation period the EZ4U test was performed and SEM investigations were revealed. The cells showed typical osteoblast-like behaviours, determined by the amount of osteocalcin and by van Kossa staining.

Osteoblasts proliferated and attached on all investigated biomaterials, except on Beriplast®P. On Ethisorb® Dura Patch the highest cell proliferation rate was measured followed by Palacos®R. Both biomaterials offer suitable growth and proliferation conditions for ovine osteoblast-like cells. SEM examinations confirmed the results of the EZ4U test. Cells cultivated on Ethisorb® Dura Patch covered the whole material surface (Fig. 4A). This is in accordance with the findings of Perka et al. [4].

The proliferation rate on Palacos®R was lower than on Ethisorb® Dura Patch, but higher than on the other investigated biomaterials. The results indicate that Palacos®R is well qualified for cell seeding. SEM showed well spread cells extending from the surface into the rough interior of the material (Fig. 4C). Normally Palacos®R, a widely available biomaterial approved for human use, acts effectively as a tissue barrier and was not used as cell culture matrix before.

The proliferation rates of the other materials (Bio-Oss®, MakroSorb®FX, PDS®-film and Titanmesh) were low, the values were between 0.019 and 0.006. SEM examinations of these materials showed less spread osteoblast-like cells and few cells with rounded morphology (not vital cells), which were sometimes almost detached from the substrate. Bio-Oss® is described on the one hand as osteoconductive, highly biocompatible material which is well qualified to be seeded with cells [34, 50]. On the other hand the properties of the material were not considerable while culturing cells on the material. Acil et al. [50] demonstrated multiple cell layers on the surface of the material by scanning electron microscopy. These findings are in contradiction with our results and the results of Wiedmann-Al-Ahmad et al. [26], where only a few attached cells were seen and where nearly no proliferation rate could be observed anymore.

MakroSorb®FX was used in animal experiments on sheep before [9]. We focused the results on the smooth surface of the material and on the amount of pores which cover the material. These conditions made the cellular inoculation onto MakroSorb®FX constructs difficult. It is proved that osteoblast-like cells attach better on rough surfaces than on smooth [42, 51]. Although MakroSorb®FX, a polylactide, owns good material characteristics as biomaterial i.e. biocompatibility and biological resorption [38], our study showed that it is not qualified for cell seeding with ovine osteoblast-like cells.

The proliferation rate on PDS®-film was also low. The surface characteristics were similar to the material MakroSorb®FX, but in addition the film had low mechanical strength, and was relatively thin (0.25 mm). The material constructs in the culture medium became uneven and

instable. This fact made the inoculation and the cellular attachment more difficult and challenging. The material was only in some areas covered with osteoblast-like cells and in other areas only uncovered, native biomaterial could be observed. This also explains the low measured cell proliferation rate on PDS®-film. In this study the PDS®-film was used for the first time as cell culture matrix. Ordinary the material was used i.e. as cell barrier [52].

The results which we reached while seeding ovine osteoblast-like cells on Titanmesh were not as good as estimated. The findings are in completely contradiction to all other results which were gained from the material titan. Titan is reported as the most biocompatible material existing and it is frequently used as graft and implant material [42, 53, 54]. We had to consider that the material titan owns very good properties as biomaterial. For our investigations we chose a commercially available mesh of titan. The mesh structure made the inoculation of the cells difficult and the inoculated cells did not attached better to the material as to the polyethylene floor of the well-plates. We concluded that cells drifted on the ground of the plates and were not capable to fix on the biomaterial again. Additionally, the titan mesh had a smooth surface which did not enhance the attachment of osteoblast-like cells.

In our study the cell cultures onto Beriplast®P showed nearly no proliferation rate. By the EZ4U test we only measured one proliferation rate on 21 Beriplast®P-constructs. Nearly no vital cells were visible on the surface of the fibrin glue. The findings are explainable because of the smooth surface of the biomaterial which affected the adherence of the cells. Our results are in contradiction with these of other authors. Perka et al. [4] combined Beriplast®P with alginate and/or Ethisorb® Dura Patch to get a more stable material. Additionally they worked with three-dimensional cell cultivation, and used an incubation period of 14 days. They described the material combinations as well qualified to be seeded with osteoblast-like cells.

Many studies demonstrated that material properties such as surface topography, surface energy and roughness, physical and chemical characteristics and material composition are important parameters to consider in the cell culture model [39].

Especially osteoblasts are sensitive to subtile differences in surface roughness and surface chemistry and to the physical characteristics of the materials with which they interact [40].

In conclusion, our study demonstrated that biomaterials with structured surfaces are more suitable for the cellular colonization and cell growth compared to unstructured surfaces independent if this surface structure is the result of roughness, porosity or material composition. Further refinement of our present investigated model involves testing a greater variety of biomaterials to determine the most suitable materials among commercially available biomaterials. The biomaterials should be composed of components that will trigger the inherent regenerative capacity of bone.

This in vitro study demonstrated the isolation and cultivation of ovine osteoblast-like cells to test different culture media and various biomaterials, and identified the best strategy to prefabricate cell seeded, osteoinductive biomaterials. Although significant advances can be made from in vitro studies, in vivo assessment of candidate bone substitutes is essential to examine biocompatibility, degradability and the potential to get quantities of bone grafts prior to clinical use.

References

1. S. J. HOLLISTER, R. A. LEVY, T. M. CHU, J. W. HALLORAN and S. E. FEINBERG, *Int. J. Oral Maxillofac. Surg.* **29** (2000) 67
2. R. MALEKZADEH, J. O. HOLLINGER, D. BUCK, D. F. ADAMS and B. S. MCALLISTER, *J. Periodontol.* **69** (1998) 1256
3. M. J. YASZEMSKI, R. G. PAYNE, W. C. HAYES, R. LANGER and A. G. MIKOS, *Biomaterials* **17** (1996) 175
4. C. PERKA, O. SCHULTZ, R. S. SPITZER, K. LINDENHAYN, G. R. BURMESTER and M. SITTINGER, *Biomaterials.* **21** (2000) 1145
5. K. G. MARRA, J. W. SZEM, P. N. KUMTA, P. A. DIMILLA and L. E. WEISS, *J. Biomed. Mater. Res.* **47** (1999) 324
6. K. WHANG, D. C. TSAI, E. K. NAM, M. AITKEN, S. M. SPRAGUE, P. K. PATEL and K. E. HEALY, *J. Biomed. Mater. Res.* **42** (1998) 491
7. A. S. BREITBART, D. A. GRANDE, R. KESSLER, J. T. RYABY, R. J. FITZSIMMONS and R. T. GRANT, *Plast. Reconstr. Surg.* **101** (1998) 567
8. M. S. BLOCK and J. N. KENT, *J. Am. Dental Ass.* **124** (1993) 139
9. G. B. CORNWALL, K. A. THOMAS, A. S. TURNER, W. R. TAYLOR and D. L. WHEELER, *Protected Bone Regeneration In An Iliac Crest Animal Model*. (Macropore, Inc., San Diego, 2003)
10. R. SCHMELZEISEN and H. SCHLIEPHAKE, *J. Craniomaxillofac. Surg.* **26** (1998) 1
11. R. SCHMELZEISEN and R. SCHÖN, *Int. J. Oral Maxillofac. Implants.* **27** (1998) 377
12. J. E. HAUSAMEN and F. W. NEUKAM, *Eur. Arch. Otorhinolaryngol.* **1** (1992) 163
13. K. E. SALYER and D. P. TAYLOR, *Clin. Plast. Surg.* **14** (1987) 27
14. S. A. WOLFE, *Clin. Plast. Surg.* **9** (1982) 539
15. D. D. LEE, A. TOFIGHI, M. AIOLOVA, P. CHAKRAVARTHY, A. CATALANO, A. MAJAHAD and D. KNAACK, *Clin. Orthop.* **367** (1999) 396
16. G. M. CRANE, S. L. ISHAUG, and A. G. MIKOS, *Nat. Med.* **1** (1995) 1322
17. A. SKOGLUND, P. HISING and C. YOUNG, *Int. J. Oral Maxillofac. Implants.* **12** (1997) 194
18. E. M. YOUNGER and M. W. CHAPMAN, *J. Orthoped. Trauma.* **3** (1989) 192
19. D. GROB, Autologous Bone Grafts. Problems at the Donor Site, edited by M. AEBI, P. REAGAZZONI, *Bone Transplantation*. (Springer, Berlin, 1989) p. 32

20. T. W. BAUER and G. F. MUSCHLER, *Clin. Orthop.* **371** (2000) 10
21. T. YOSHIKAWA, H. NAKAJIMA, E. YAMADA, M. AKAHANE, Y. DOHI, H. OHGUSHI, S. TAMAI and K. ICHIJIMA, *J. Bone Miner. Res.* **15** (2000) 1147
22. P. DUCHEYNE and Q. QIU, *Biomaterials* **20** (1999) 2287
23. H. E. SCHRATT, G. REGEL, B. KIESEWETTER and H. TSCHERNE, *Unfallchirurg* **99** (1996) 679
24. R. C. THOMSON, A. G. MIKOS, E. BEAHM, J. C. LEMON, W. C. SATTERFIELD, T. B. AUFDEMORTE and M. J. MILLER, *Biomaterials* **20** (1999) 2007
25. G. B. STARK, R. HORCH and E. E. TANCZOS, *Biological Matrices And Tissue Reconstruction*. (Springer-Verlag, Berlin Heidelberg New York, 1998)
26. M. WIEDMANN-AL-AHMAD, R. GUTWALD, N.-C. GELLRICH, U. HÜBNER and R. SCHMELZEISEN, *J. Mater. Sci. Mater. Med.* **16** (2005) 57
27. U. SCHNEIDER, *Orthopäde* **27** (1998) 143
28. U. RIPAMONTI, J. CROOKS and D. C. RUEGER, *Plast. Reconstr. Surg.* **107** (2001) 977
29. H. TERHEYDEN, C. KNAK, S. JEPSEN, S. PALMIE and D. R. RUEGER, *Int. J. Oral. Maxillofac. Surg.* **30** (2001) 373
30. O. HANISCH, D. N. TATAKIS, M. D. ROHRER, P. S. WOHRLE, J. M. WOZNEY and U. M. WIESJO, *Int. J. Oral Maxillofac. Implants.* **12** (1997) 785
31. T. UEMURA, J. DONG, Y. WANG, H. KOJIMA, T. SAITO, D. IEJIMA, M. KIKUCHI, J. TANAKA and T. TATEISHI, *Biomaterials.* **24** (2003) 2277
32. R. ZHANG and P. X. MA, *J. Biomed. Mater. Res.* **44** (1999) 446
33. K. J. BURG, S. PORTER and J. F. KELLAM, *Biomaterials.* **21** (2000) 2347
34. S. S. JENSEN, M. AABOE, E. M. PINHOLT, E. HJORTING-HANSEN, F. MELSEN, and I. E. RUYTER, *Int. J. Oral Maxillofac. Implants* **11** (1996) 55
35. D. CAMPOCCIA, C. R. ARCIOLA, M. CERVELLATI, M. C. MALTARELLO and L. MONTANARO, *Biomaterials.* **24** (2003) 587
36. N. R. KÜBLER, *Mund Kiefer GesichtsChir.* **1** (1997) 2
37. J. DOBKOWSKI, R. KOLOS, J. KAMINSKI and H. M. KOWALCZYNSKA, *J. Biomed. Mater. Res.* **47** (1999) 234
38. S. L. ISHAUG, G. M. CRANE, M. J. MILLER, A. W. YASKO, M. J. YASZEMSKI and A. G. MIKOS, *J. Biomed. Mater. Res.* **36** (1997) 17
39. K. ANSELME, M. BIGERELLE, B. NOEL, E. DUFRESNE, D. JUDAS, A. LOST and P. HARDOUIN, *J. Biomed. Mater. Res.* **49** (2000) 155
40. B. D. BOYAN, T. W. HUMMERT, D. D. DEAN and Z. SCHWARTZ, *Biomaterials* **17** (1996) 137
41. B. D. BOYAN, V. L. SYLVIA, D. D. DEAN, C. H. LOHMANN, D. L. COCHRAN and Z. SCHWARTZ, *Osteologie.* **8** (1999) 123
42. G. LAUER, M. WIEDMANN-AL-AHMAD, J. E. OTTEN, U. HÜBNER, R. SCHMELZEISEN and W. SCHILLI, *Biomaterials* **22** (2001) 2799
43. W. TAN and T. A. DESAI, *Tissue Eng.* **9** (2003) 255
44. J. A. FIALKOV, C. E. HOLY, M. S. SHOICHET and J. E. DAVIES, *J. Craniofac. Surg.* **14** (2003) 324
45. P. TORRICELLI, M. FINI, G. GIAVARESI, M. ROCCA, G. PIERINI and R. GIARDANO, *J.Biomed. Mater. Res.* **52** (2000) 177
46. D. W. HUTMACHER, *Biomaterials* **21** (2000) 2529
47. P. WATTS, M. D. SMITH, I. EDWARDS, V. ZAMMIT, V. BROWN and H. GRANT, *J. Hepatol.* **23** (1995) 605
48. I. GERBER and I. AP GWYNN, *Eur. Cells Mater.* **2** (2001) 10
49. M. WIEDMANN-AL-AHMAD, R. GUTWALD, G. LAUER, U. HÜBNER and R. SCHMELZEISEN, *Biomaterials* **23** (2002) 3319
50. Y. ACIL, H. TERHEYDEN, A. DUNSCHE, B. FLEINER and S. JEPSEN, *J. Biomed. Mater. Res.* **51** (2000) 703
51. F. GRIZON, E. AGUADO, G. HURE, M. F. BASLE and D. CHAPPARD, *J. Dent.* **30** (2002) 195
52. M. CHRISTGAU, N. BADER, A. FELDEN, J. GRADL, A. WENZEL and G. SCHMALZ, *J. Clin. Periodontol.* **29** (2002) 710
53. A.W. SUGAR, M. KURIAKOSE and N. D. WALSHAW, *Int. J. Oral Maxillofac. Surg.* **21** (1992) 140
54. M. B. HÜRZELER, C. R. QUINONES, A. KIRSCH, C. GLOKER, P. SCHÜPACH, J. R. STRUB and R. G. CAFFESSE, *Clin. Oral Impl. Res.* **8** (1997) 467