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Implant Surface Coatings with Bone Sialoprotein, Collagen, and Fibronectin and their Effects on Cells Derived from Human Maxillar Bone

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Abstract
The interaction between implant material and surrounding tissues is believed to play a fundamental role in implant success. Although bone sialoprotein (BSP) has been found to be osteoinductive when coated onto femoral implants, collagen and fibronectin are the most used compounds for preparation of precoated cell culture slides at present.

In this study, the support of BSP-, collagen- and fibronectin-coated and non-coated implant material for the development of adult human maxillar bone *in vitro* was studied and compared. The expression of bone turnover markers like BSP and osteocalcin as well as osteonectin, transforming growth factor beta (TGF-beta) and CD90 during different time periods of cell cultivation (3, 5, 10, 15, 20 and 25 days) was visualized immunohistochemically. The distribution patterns of the cells were examined on a rough surface of the titanium-hydroxyapatite dental implant material TICER and on a total smooth surface of the technical implant material glimmer.

Significantly different values were found for glimmer at the 15. and the 20. Div, exclusively, indicating that a smooth surface was more improved than a rough ceramic surface by pre-coatings. The White-test using rankings of the median values gave evidence for BSP-coatings at position 1 followed by collagen.

Our experiments were designed to use very low concentrated BSP coating solution with the aim to reduce the healing time with a minimal effort and minimal risks for the patients.

Key words: Bone, implants, non-collageneous proteins

Introduction

Aseptic loosening of implants remains an unsolved orthopedic problem. The interaction between implant material and surrounding tissues is believed to play a crucial role in implant success or failure. Adaptive cellular responses to the implant material include alterations in the cytoskeleton, integrin expression and synthesis of extracellular matrix proteins. Beside osteocalcin and osteonectin, bone sialoprotein (BSP) is the major non-collagenous protein of the extracellular bone matrix (Fisher 1985; Fisher and Termine 1985; Marks and Popoff 1988). BSP is a highly glycosylated and sulphated phosphoprotein with a molecular weight of 70-80 kDa (Ganss et al. 1999) that is found almost exclusively in mineralized connective tissues. Characteristically, polyglutamic acid and arginine-glycine-aspartate (RGD) motifs with the ability to bind hydroxyapatite and cell-surface integrins, respectively, have been conserved in the protein sequence of different species. BSP expression coincides with initial bone mineralization and it serves as a center of crystallization for hydroxyapatite (Hunter and Goldberg 1993; 1994; Hoshi and Ozawa 2000). Furthermore, expression of the BSP gene, which is induced in newly formed osteoblasts, is up-regulated by hormones and cytokines that promote bone formation, e.g. transforming growth factor-beta (TGF-beta). In addition, the morphological induction and maintenance of hydroxyapatite nucleation during bone formation has been shown to correlate with TGF-beta expression.

Recently, O`Toole et al. (2004) demonstrated that BSP is osteoinductive when coated onto femoral implants. On the other hand, osteoblasts have been found to adhere on BSP-coated surfaces in the similar way as on collagen- and fibronectin-treated ones.

Therefore, the aim of the present study was to compare BSP-, collagen- and fibronectin-coated and non-coated implant material in enhancing the development of adult human maxillar bone at different time intervals: 3, 5, 10, 15, 20 and 25 days *in vitro* (Div). As the cascade of bone cell differentiation is initiated by the TGF-beta supergene family and develops as a mosaic structure, antibodies against TGF-beta, BSP, osteocalcin and osteonectin were used to characterize osteoblast-like cells. For immunohistochemical staining of fibroblasts and non-differentiated osteoblasts, the antibody against human CD90 was applied.

Material and Methods

Tissue Preparation

All procedures used in the present study were approved by the Ethics Committee of the University of Leipzig. The rules of the Declaration of Helsinki 1964

(NIH publication no. 86-23, revised 1985) were followed. Human maxillar bone samples without any clincal or radiographic evidence of pathology were obtained from two male donors (40 and 45 years old) undergoing dental surgery at the Department of Oral-, Maxillo-, Facial- and Reconstructive Plastic Surgery, University Hospital of the University of Leipzig. In each case, the bone samples were placed in a sterile tube containing sterile 0.05 M phosphate buffered saline (PBS), pH 7.4, and penicilline/streptomycine, 10 000 IU/ml each (PromoCell, Germany). Thereafter, all samples were processed under sterile conditions. The maxillar bones were cut in 0.1-0.2 cm pieces. After several rinses in PBS, the material was incubated with 0.25% collagenase type IV (166 U/mg, Biochrom AG, Germany) for 3 hours at 37 °C, washed and cultured in Dubelcco´s modified Eagle´s medium (PromoCell, Germany) supplemented with 10% fetal bovine serum (PromoCell, Germany) in an atmosphere of 5% CO_2 – 95% air at 37 °C. The medium was changed twice weekly. Cells were subcultured from the initially isolated primary cells and seeded at a density of 4000 cells/well in six-well plates. In all experiments cells were used at first passage.

Implants

The dental implant material TICER and the technical material glimmer were used in the experiments. TICER (ZL-Microdent, Germany), based on a material developed by Graf (1997) and described by Schreckenbach et al. (1999), consists of titanium with hydroxyapatite ceramic and has a roughened surface to improve the attachment, spreading, and growth of the bone cells on the metal implant (Hulbert et al. 1970; Schwartz et al. 1999). In contrast, glimmer has a total smooth surface. The implant samples with a size of 10 x 30 x 1mm were coated with BSP, collagen and fibronectin as follows: 10µg of BSP (His-Myc-Ek-BSP, Immundiagnostik, Bensheim, Germany) were dissolved as described by Wuttke et al. (2001), and 10µg of fibronectin (Biochrom, Germany) in 1 ml of 0.9M sterile PBS, pH 7.2. Collagen (Cohesion, USA) was used as VITROGEN 1% (bovine dermal collagen dissolved in 0.012 N HCl). Then twenty-four implants were incubated for two hours in 300 µl of BSP, fibronectin or collagen solution. The treated implants were removed from the coating solutions and allowed to dry under sterile conditions for 12 hours at room temperature. Thereafter, the coated implants were placed in six-well cell culture plates, the cells were seeded and grown for different days (3, 5, 10, 15, 20 and 25) *in vitro*.

Immunocytochemistry

Cells were fixed with 4% PBS buffered paraformaldehyde for 15 minutes and rinsed in PBS. For immunohistochemical characterization, cells on the surface of the implant discs were treated for two hours with a blocking agent, 10% normal goat serum (Vector, Burlingame, USA) or a mixture of 10% normal goat and 10% normal donkey serum in PBS, and incubated overnight at 4 °C with 1:100 diluted primary antibodies against BSP (monoclonal mouse anti-human BSP, Immundiagnostik, Bensheim, Germany), osteocalcin (monoclonal, rabbit-anti-human, Acris, Hiddenhausen, Germany), osteonectin (monoclonal, mouse-anti-human, Acris), TGF-beta (monoclonal, rabbit-anti-human, Chemicon, Temecula, USA) and CD90 (monoclonal anti-human CD90, FITC-conjugated, DIANOVA, Hamburg, Germany) according to Saalbach et al. (1997). For simultaneous detection of BSP and CD90 or osteonectin and TGF-beta, implants were treated with a mixture of the corresponding antibodies. After washing in PBS, the bound primary mouse antibodies were visualized by incubation with 1:50 diluted goat-anti-mouse-Cy3 (Jackson Immuno Research) in 4% PBS buffered bovine serum albumin (Serva, Germany) for two hours, whereas for visualization of the primary rabbit antibodies a donkey-anti-rabbit-Cy2 (Jackson Immuno Research) was used. After several rinsings with PBS, the cell preparations were counterstained using DAPI (Serva, Heidelberg, Germany), dried and coverslipped. Control sections were put through the same procedure with nonspecific mouse or rabbit antibodies (DAKO).

Immunostained cell preparations on the implant surfaces were analyzed using a motorized Zeiss Axiophot2 microscope equipped with appropriated filters. Separate images for DAPI-, Cy3-, Cy2- and FITC-labeled cells were recorded digitally as color-separated components using an AxioCam digital microscope camera and AxioVision multi-channel Image processing (Zeiss Vision GmbH, Germany). Individual color images (blue for DAPI, red for Cy3, green for Cy2 and FITC) were merged, and the co-expression sites appeared in white-yellow color. Representative images of the experiments are shown in Figs. 2 and 3.

Statistical Analysis

DAPI-BSP, DAPI-osteocalcin, and DAPI-osteonectin labeled cells were counted on the borderline as well as in the middle of the corresponding implant surface. Three to ten successive sections were screened at 400x magnification on a frame of 350µm x 230µm. Only the values of analogous parts were used for averaging. The interactive measurements were carried out using the software of the Kontron-Videoplan-System (Kontron, Zeiss, Germany) and the "frozen image tool". The system software of the Videoplan provided the mean (average ± standard deviation) and the median values for each file. The U-test was used to compare the data sets of the different files and to determine the level of significance of the differences between the various coated surfaces. For a rapid approximation, ranks in a test of Wilcoxon (1945) and White (1952) were used.

AFM Microscopy

The atomic force microscope (AFM) moves a very small sharp tip attached to a soft cantilever, which acts as a spring, in a raster pattern over the sample surface. Deflections in the tip corresponding to surface topography are recorded. The AFM can be operated in air and liquids. In routine diagnosis, it is often desirable to

minimize cantilever deflections in order to protect the sample from too large and potentially damaging forces. For this procedure, a feedback loop is used to adjust the sample height while imaging. The analysis was performed on an AFM (Topometrix Explorer) with 130 μm xy-scan range and 10 μm z-scanner. The AFM is mounted on the top of an inverted microscope (Zeiss Axiovert 135) in order to select the region of interest on the sample surface. For observations of the specimen under ambient conditions, soft cantilevers were utilized in constant force mode (sharpened microlevers, spring constant = 0.02 N/m, tip radius < 10 nm, Thermomicroscopes Sunnivale CA USA). The forces applied during AFM measurements were 10-20 nN under ambient conditions (Thalhammer et al. 2001).

Results

Morphology

The BSP expression in cells settled on collagen- and BSP-coated TICER surface (red color) was first detectable at day 5 *in vitro* (Fig. 2). Some of the cells were extremely large sized, especially at the BSP-coated implant surface. BSP was mainly localized within the non-mineralized matrix. Although at the beginning of the experiment the morphology of the cells was spheroidal, after one day it changed to the typical polygonal one (not shown). The CD90 marker (green color) indicated a co-localized expression of BSP and CD 90. In the control sample (Fig. 2), the dominance of the green color persisted until day 20 *in vitro*. The expression of osteocalcin and osteonectin was similar to that of BSP, whereas TGF-beta differed significantly. To a greater extent these features were seen also on the glimmer surface as shown in Fig. 3 for day 5 *in vitro*. The distribution patterns of cells on the implant surfaces varied widely as revealed using DAPI staining of nuclei. BSP expression in cells settled on the TICER surface displayed a half-circular pattern at days 10 and 15 *in vitro* (Fig.2). At days 20 and 25 *in vitro*, cells were homogeneously distributed on the implant surface including a random sample.

Cell Counts

Cell counts revealed that most of the survived cells are settled on the surface of the BSP-coated implants. Fig. 4 displays the median values and the standard deviations indicating only small differences between the various samples. Significantly different values were found at days 15 and 20 *in vitro*, especially for glimmer surfaces. Ranking of the median values gives evidence for BSP-coatings at position 1 followed by collagen. All glimmer samples, coated and non-coated, exhibit significantly smaller standard deviations in the number of settled cells than the TICER ones. In summary, the observed promotion of cell settlement and/or enhanced cell proliferation on the TICER surface was not significant.

The control experiment using non-coated materials revealed that TICER is superior in number of adhered cells, especially at day 25 *in vitro*. That superiority was changed by pre-coating with BSP solution.

Atomic Force Microscopy

The results of the atomic force microscopy revealed that the surface patterns of BSP-, collagen- and fibronectin-coated glimmer differed widely (Fig. 1, left column). The best results were found for collagen promoting cell settlement on the glimmer samples with a total homogeneous distribution pattern covering the whole surface. The BSP- coating results in very few drops of the BSP solution on the surface. Some more drops on the surface were revealed for fibronectin coating with the highest level of the z range. It can be summarized, that cells with different soma sizes and features were found, whereas the distribution patterns of the settled cells were identical for all coating types (Fig. 1, right column). The influence of the BSP coating on the glimmer surface with discontinuously moistened surface is shown in Fig. 5 which displays the borderline (see Fig. 1, BSP left column) of the coating. Here a disruption of cell number and BSP expression as well as cell features were revealed. Comparison of cell distribution patterns on the glimmer surface with these on the dental implant material surface demonstrates that cell attachment depends to a higher degree on the surface design than on its coating. In materials with identically designed surfaces, BSP-coating promotes cell settlement to a higher degree than the other used coating materials.

As a conclusion, we found 1) a benefit of BSP-coated smooth implant surfaces for the osteoinduction; 2) an enhancement of the osteoinductive effect of surface roughness by BSP-coating.

Discussion

Successful attachment on artifical surface is prerequisite for inducing new bone formation locally at the site of implantation. Protein-coated surfaces may influence the biocompatibilty of implant materials by initiating and supporting osteogenesis (Sodek and McKee 2000). Collagen, fibronectin, vitronectin or mixtures of natural extracellular matrix proteins are the mostly investigated proteins for this purpose (Meyer et al. 1998; Lacouture et al. 2002; Salih et al. 2002). BSP was found to be osteoinductive in bone repair (Wang et al. 2004) and sufficient to achieve healing in critical defects (Wang et al. 2002). Lacouture et al. (2002) compared type I collagen, fibronectin, and vitronectin in supporting adhesion of mechanically strained osteoblasts and found out that the major factor governing strain resistance was the number of integrin-extracellular matrix attachments when the number of molecules available for attachment was limited. At a low concentration of protein-coating density, collagen supported the highest attachment rate followed by fibronectin and vitronectin. At higher concentrations, vitronection supported the highest attachment rate after 24 hours *in vitro*.

Nevertheless, the key of the success of implants is related to the initial healing process (Lekic et al. 1996). Most of the authors (Cook et al. 1985; Kieswetter et al. 1996; Leize et al. 2000) suggest that the larger surface area of rough material leads to an initially delayed, but then prolonged cell proliferation. The initial delay

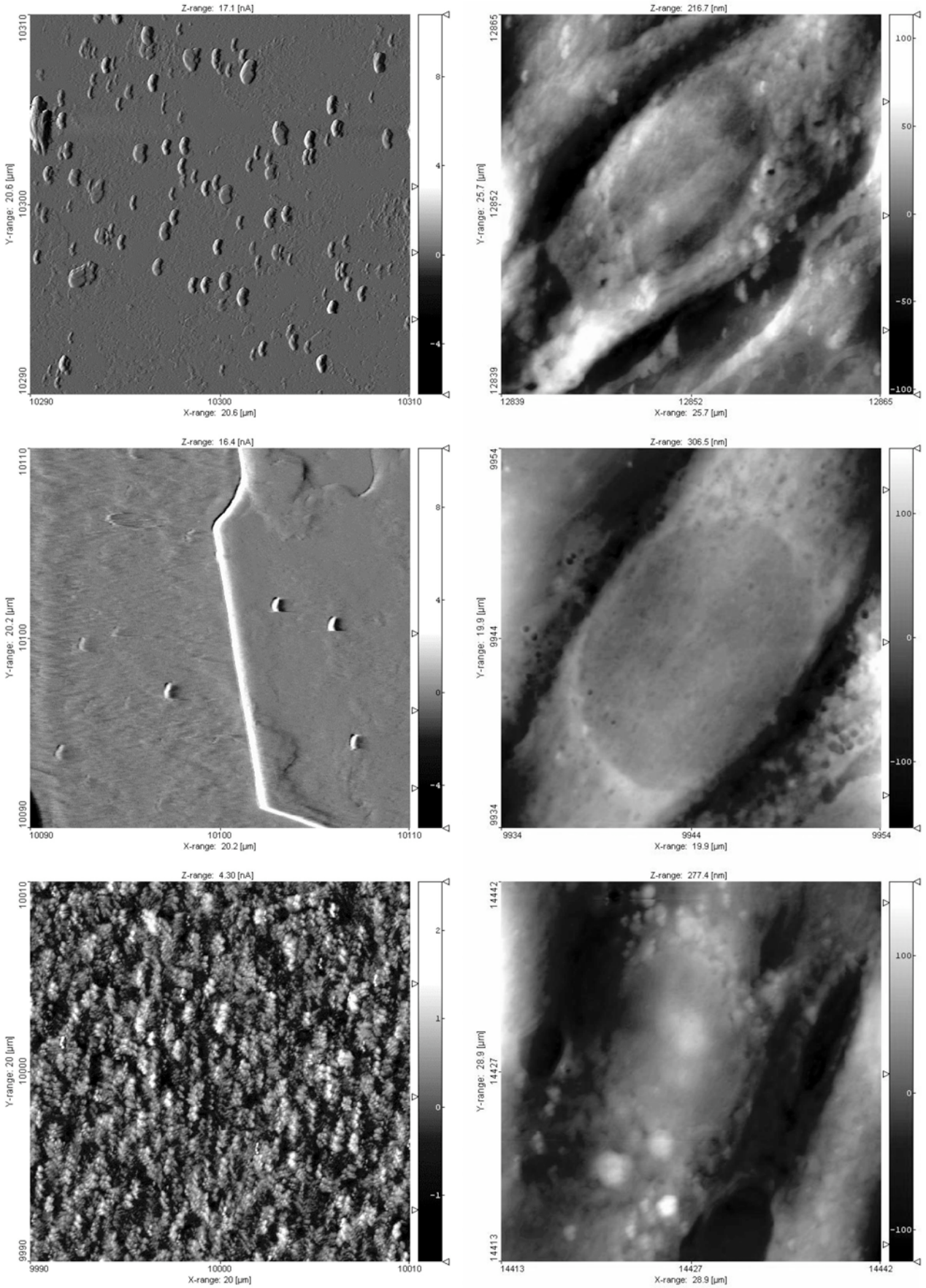


Fig. 1. Left column: Atomic force microscopic features of the glimmer surface coated with fibronectin (above), BSP (middle) and collagen (bottom). Right column: Features of the settled cells at the glimmer surface coated with fibronectin (above), BSP (middle) and collagen (bottom).

Fig. 2

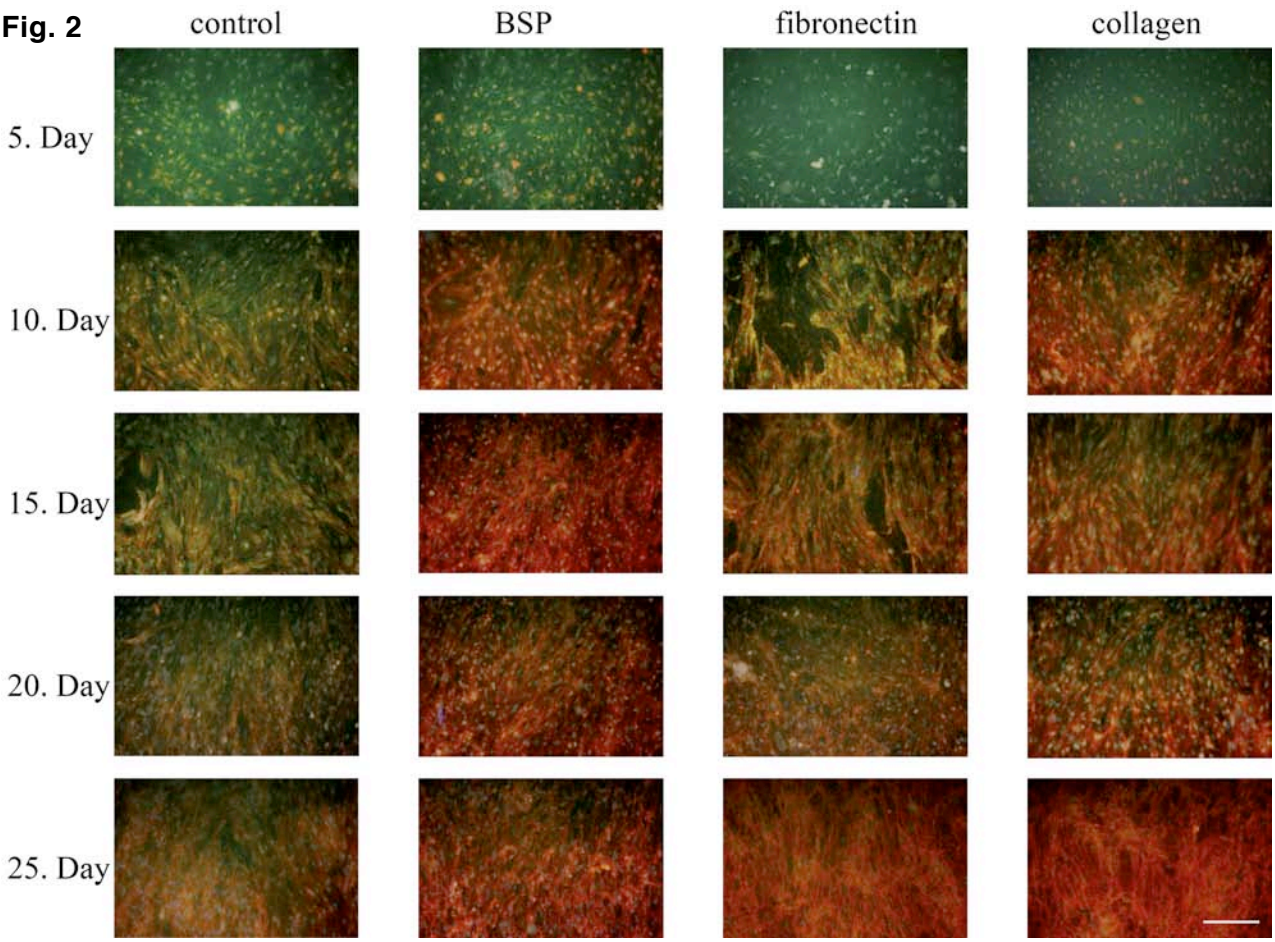
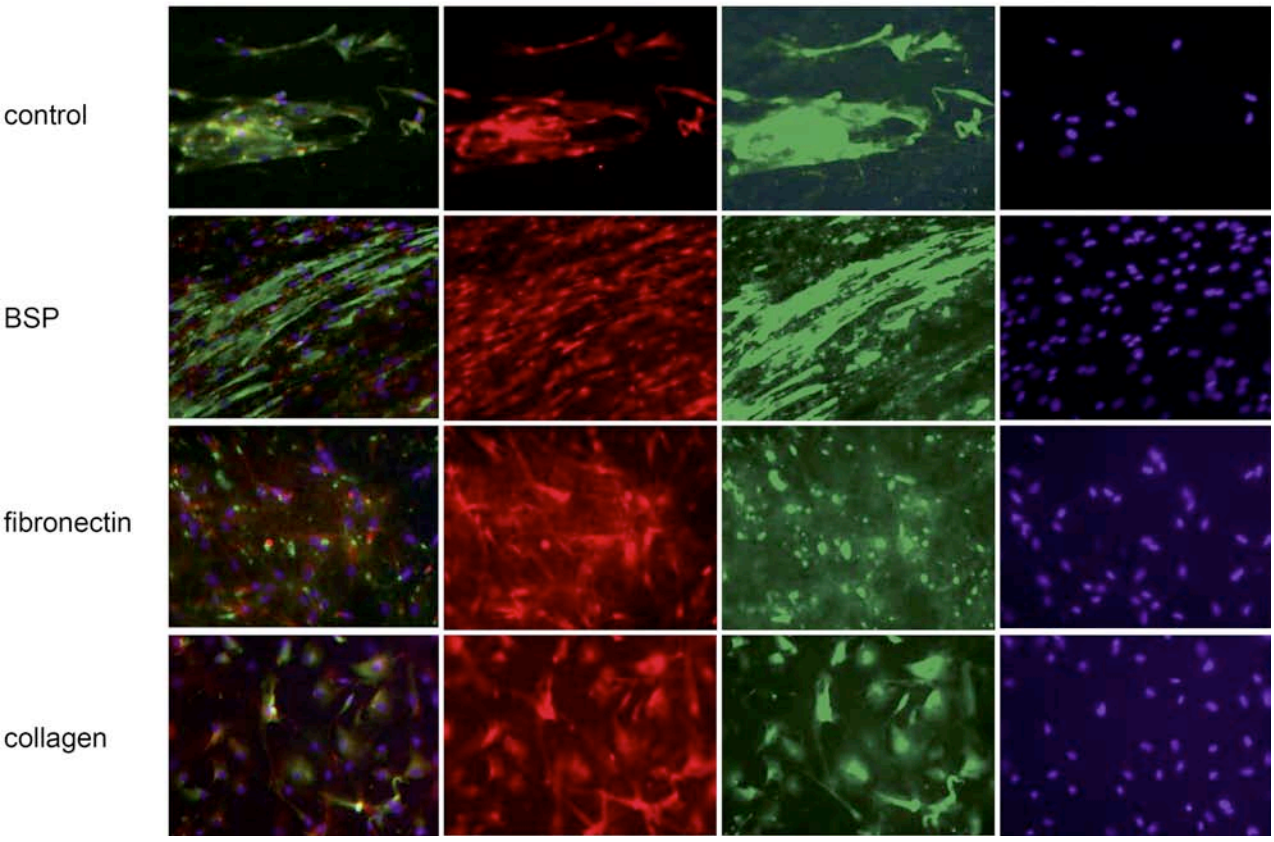

control
BSP
fibronectin
collagen
5. Day
10. Day
15. Day
20. Day
25. Day

Fig. 3


merged
osteonectin
TGF-beta
DAPI
control
BSP
fibronectin
collagen

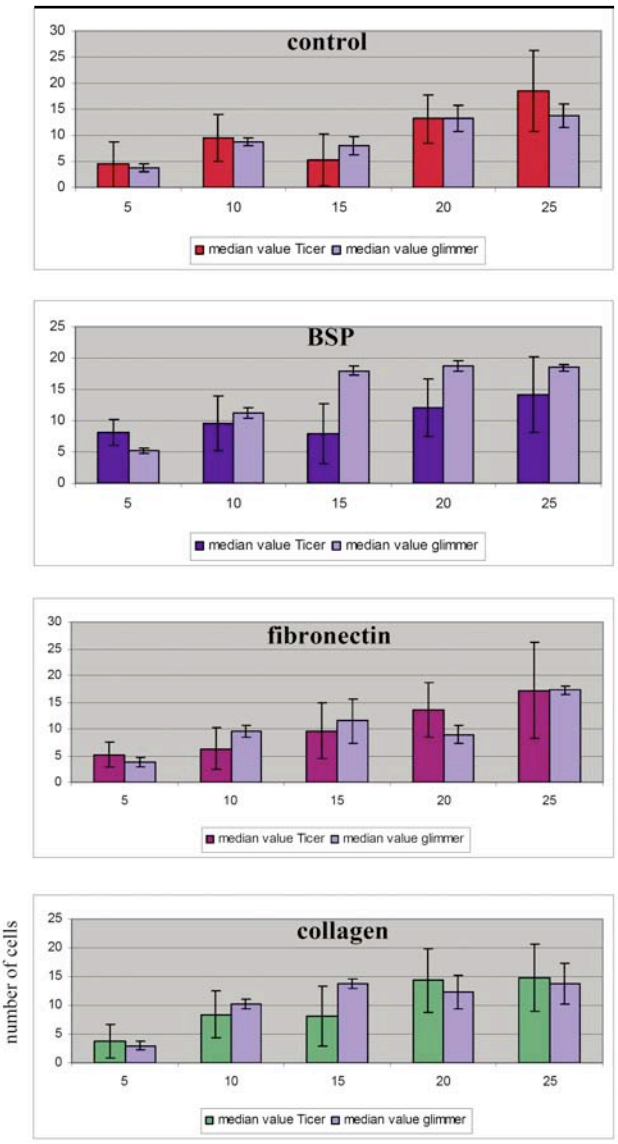


Fig. 4. Development of the number of cells settled the pre-coated surfaces from day 5. to day 25. *in vitro*.

at the rough TICER surface can be compensated by BSP-coated surface. The deposition of BSP represents the first step of bone formation in ectopic transplantation systems in vivo (Riminucci et al. 2003). Chou et al (2005) reported the expression of BSP in relation to the provided inductive agents to the cell culture. That fact was supported by our results. The clinical superiority of human derived BSP (when compared with bovine collagen) based on the better histocompatibility of the former.

Our experiments were designed to use very low concentrated BSP coating solution with the aim to reduce the healing time with a minimal effort and risks for the patients.

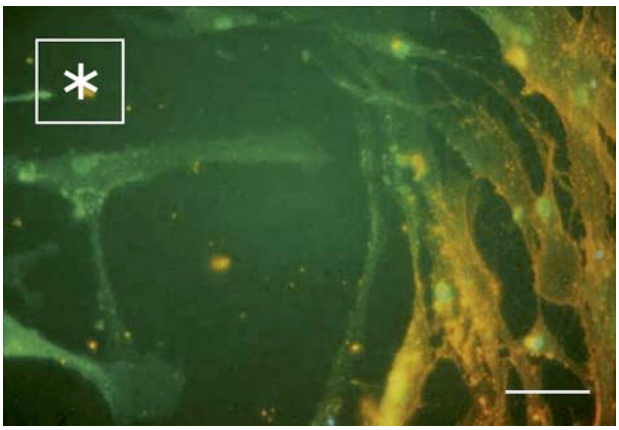


Fig. 5. Effect of coating visualized at the coating border on the glimmer surface by lacking BSP expression of settled cells, * indicates the part of the sample without BSP coating. Bar: 50μm.

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◀ *Fig. 2*. Expression of BSP (red immunofluorescence=BSP, blue=DAPI=nuclei of cells) and patterns of CD90 (green) in cells settled on the pre-coated surface of the implant material TICER. Bar: 100μm.

◀ *Fig. 3*. Expression of osteonectin and TGF-beta in cells settled on pre-coated surface of glimmer at day 5 *in vitro* (DAPI= nuclei of cells). Bar: 50μm.

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