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Effect of biological implant surface coatings on bone formation, applying collagen, proteoglycans, glycosaminoglycans and growth factors

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Abstract
Objectives The aim of the present study was to evaluate six different implant surface coatings with respect to bone formation. Being major structural components of the extracellular matrix, collagen, the non-collagenous components decorin/chondroitin sulphate (CS) and the growth factors TGF-β1/BMP-4 served in different combinations as coatings of experimental titanium implants.
Materials and methods Eight miniature pigs received each six implants in the mandible. The implant design showed two circular recesses along the length axis. Three, four, five and six weeks after implant placement, the animals were sacrificed in groups of two. Bone-implant contact (BIC) was evaluated along the outer implant surface and within the recesses. Bone volume was determined by synchrotron radiation micro computed tomography (SRμCT) for one implant of each surface state, 6 weeks after placement.
Results At each week of observation, collagen/CS or collagen/CS/BMP-4 coated implants showed the highest BIC of all surface states. This was statistically significant at week five ($p = 0.030$, $p = 0.040$) and six ($p = 0.025$, $p = 0.005$). SRμCT measurements determined the highest bone volume for a collagen/CS coated implant.
Conclusion The results indicate that collagen/CS and collagen/CS/BMP-4 lead to a higher degree of bone formation compared to other ECM components.

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Introduction

The use of endosseous implants has gained acceptance and became a routine clinical treatment in dental surgery, generally achieving good results. Given normal bone formation the long-term implant success and survival rates are above 90% [1–3]. However, problems still exist in cases of low bone density and quantity [4]. This can be caused by systemic disorders like osteoporosis or be encountered as a side effect of radiotherapy. Such circumstances imply a challenging bone healing situation. Under these conditions the survival rate of dental implants may decrease to 55% [5, 6]. One of the main fields of investigation is to facilitate bone formation and to improve bone response to titanium implants. This demands for new approaches to implant osseointegration.

The integration of an implant is determined to a large part by the interaction of cells with the implant surface, which influences cellular responses and ultimately the newly formed tissue. An important component of the cellular environment is the extracellular matrix (ECM), which is composed of collagens, glycoproteins, proteoglycans and glycosaminoglycans, assembled locally in an ordered, highly site-specific network. To improve the biocompatibility of implant materials, one approach is to apply coatings consisting of ECM proteins.

Adhesion to the ECM is intimately coupled to signal transduction and most adhesion receptors function as sig-

nalling molecules. Engagement of these different receptors gives rise to a wide variety of intracellular signals, often acting synergistically with signals derived from growth factor receptors [7–9]. Another function of the ECM in vivo is that of a reservoir for growth factors, influencing their activity by binding and presenting them [10–13]. The ECM thus not only provides cells with a scaffold for adhesion, but also takes an active part in regulating the cellular processes and responses.

Combining growth factors with specific ECM elements in implant coatings utilizing ECM components that can interact both with collagen and growth factors in analogy to the situation in vivo may serve to enhance the growth factor activity and thus reduce the necessary amounts. Decorin, a small leucin rich protein with a core protein (~50 kD) glycosilated with either a dermatan sulphate or, in bone, a chondroitin sulphate (CS) chain (~70 kD)—is described as influencing binding and activity of transforming growth factor β1 (TGF-β1), as well as binding to collagen fibers both in vivo and in vitro [14–16]. For glycosaminoglycans alone such interactions are also described: i.e., heparan sulphate with fibroblastic growth factor (FGF) [17–19] or CS with bone morphogenetic proteins (BMP) (Bierbaum, in preparation). For this reason CS 4, a glycosaminoglycan (GAG) consisting of a repeating disaccharide unit of glucuronic acid and *N*-aminoacetyl-galactosamine-sulphate, was of special interest in this study. Both ECM components can be integrated into the collageneous coating of implants, giving rise to changes in matrix morphology [20].

Another aspect that may serve to enhance growth factor activity are synergistic effects, for instance between BMP and TGF. TGF also plays a pivotal role in bone metabolism, affecting among other things cell proliferation [21]. As BMP influences the differentiation of the cells, the combination of both factors may potentiate their effects [22–24].

The main aim up to now has been to provide cells with a surface more suited to adhesion [25–27]. For titanium implants it could be shown that such coatings increase the rate of bone apposition over an uncoated reference material significantly [7].

To this end implant coatings consisting of a collagenous matrix, being modified using non-collagenous components with a potential to bind BMP-4 and TGF-β1 (CS and decorin) were examined, both with and without the growth factors BMP-4 and TGF-β1.

The aim of this animal study was to investigate whether implants, coated with further components of the ECM, could improve bone formation, compared to collagen coated implants. Bone formation at different times of interest was accessed, measuring the bone-implant contact (BIC) histomorphometrically. Further bone volume was analysed by SRμCT measurements.

Materials and methods

Reagents

Employed components for surface coatings were acid soluble bovine skin collagen type I (Fluka, Deisenhofen, Germany), CS from bovine trachea and decorin from bovine articular cartilage (Sigma, Germany). Human recombinant growth factors BMP-4 and TGF-β1 were obtained from R&D (Wiesbaden-Nordenstadt, Germany).

Design and coating of implants

Cylindrical titanium implants with a diameter of 4 mm and a length of 12 mm were used in this study. These specially designed implants were based on a Xive© (Frialit, Germany) implant. The implant geometry included two recesses along of the implant axis to create a defined area between the implant surface and the round drill hole. These recesses were designed with a defined width of 2.50 mm and a depth of 0.375 mm for the upper and 0.875 mm for the apical recess. The titanium implants were sandblasted with 250 µm corundum and cleaned with 1% Triton X-100, acetone, and 96% ethanol, rinsed with distilled water, and air dried.

The generated surfaces states were:

(i) collagen (coll)
(ii) collagen + decorin (coll/DC)
(iii) collagen + chondroitine sulphate (coll/CS)
(iv) collagen + decorin + TGF-β1 (coll/DC/TGF)
(v) collagen + chondroitin sulphate + BMP-4 (coll/CS/BMP)
(vi) collagen + chondroitin sulphate + decorin + TGF-β1 + BMP-4 (coll/DC/CS/BMP/TGF)

Collagen was dissolved at 5 mg/mL in 10 mM acetic acid over night at 4 °C. The collagen solution was then mixed on ice with equal volumes of twofold concentrated fibrillogenesis buffer (60 mM sodium phosphate, 270 mM NaCl, pH 7.0). CS and decorin were added to 30 µg/1 mg collagen. Fibrillogenesis was allowed to take place overnight at 37 °C. The resulting gel was homogenized, fibrils were collected by centrifugation at 5,000 × *g* for 15 min, washed with fibrillogenesis buffer diluted to working concentration, and centrifuged again. The pellet was resuspended in the same buffer to a concentration of about 5 mg/mL collagen. The implants were incubated in the suspension at 25 °C for 5 min and air dried. This process was repeated two times; the coated implants were then washed with distilled water and sterilized with ethylene oxide at 42 °C for 12 h.

Growth factors were allowed to adsorb to the surfaces over night at 4 °C with 100 ng/mL TGF-β1 and 2 µg/mL BMP-4.

Fig. 1 Coated titanium implant with two recesses along the length axis

Surfaces were characterized by scanning electron microscopy at 1 kV acceleration voltage with a DSM 982 Gemini (Leo GmbH, Oberkochen, Germany) (Fig. 1).

Surgical procedure

In this study eight one year old miniature pigs with an average weight of 70 kg have been operated. The animals were housed in a stable on the countryside. In a first operation the primary premolar teeth were extracted and the permanent tooth germs removed. All surgical procedures and medical examinations were performed under general anaesthesia. For sedation, 1 mg/kg body weight midazolam and 10 mg/kg body weight ketamine were injected intramuscularly. To reduce salivation, 0.05 mg/kg body weight atropine was added to the injection. Anaesthesia was induced by an injection of 2–4 mg/kg body weight carprofen. To reduce the pain after surgery, all minipigs received Durogesic© (fentanyl) postoperatively. Eight month after primary premolar removal and germectomy, a second surgery was performed. 48 implants were placed endosseously in the lower jaw under the antibiotic coverage of 5 mL/kg body weight Duphamox LA© (amoxicillin), applied as an intramuscular injection. Each minipig received 6 implants (1 of each coating) in a randomized trial. The implants were covered by a cover screw before the soft tissue was sutured.

In order to compare the degree of osseointegration in between the surface coatings, different times of interest were selected. The minipigs were sacrificed in groups of two by an overdose of T 61© (embutramid, mebezonium iodide, tetracain) at 3, 4, 5 and 6 weeks after implantation. After euthanasing the animals, the implants were removed in block section for volume measurement and histomorphometric investigation of bone-implant contact.

SR μCT

After scarifying the pigs, implants and surrounding bone sections where removed and embedded in Technovit 9100 neu© (Heraeus-Kultzer, Wehrheim, Germany). Cylindrical samples (one sample per surface state) were prepared with a diameter of about 8 mm, containing the implant nearly centred within the bony tissue. Six implants, (one sample per surface state from the 6 weeks group) were analysed by SRμCT.

The measurements were performed at HASYLAB BW5 (DESY, Hamburg, Germany). With a photon energy of 70 keV and an image size of 1,024 × 1,024 pixels 720 projections per implant were recorded. A filtered back projection algorithm was used to obtain the three-dimensional data of X-ray absorption for the samples. The visualization of the reconstructed data was done with a volume rendering software (VGStudio, Volume Graphics, Germany). Automatic and semi-automatic analysis procedures were created to record the amount of mineralized bone in SRμCT volume. The volume was measured within the area, defined by the lower implant recess. This volume was compared to a region of reference, being defined by an area half the size of the recess, bordering in implantofugal direction. Thus, an increase or a decrease in volume within the recess, compared to the region of reference could be determined.

The samples could later be prepared for the histomorphometric analysis (Fig. 2).

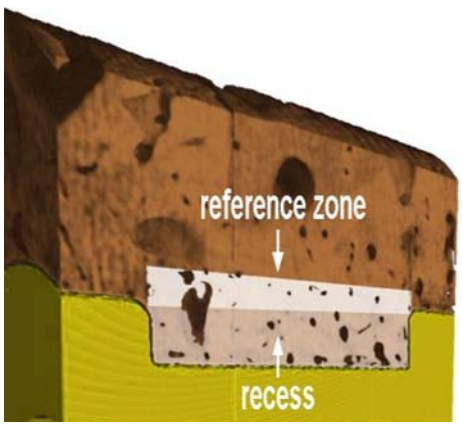

Fig. 2 Scheme of the implant recess and the reference zone in an osseointegrated state: region of interest for SRμCT bone volume measurements

Histomorphometry

Undecalcified sections were cut sagitally along the axis of each implant using a diamond saw microsectioning system (Exakt-Apparatebau, Norderstedt, Germany). Three to five sections could be gained per implant. These sections were reduced to 30 µm, using Donaths grinding technique on a roll grinder containing sandpaper [28].

As a next step, the histological sections were stained according to Masson-Goldner. For each section, the implant surface length in contact with osseus tissue was calculated. It was expressed as the percentage of the total surface length of the implant. In a second measurement the same parameter was measured within the recesses. The samples were analysed using transmitted light microscopy (BX 61, Olympus, Hamburg, Germany) connected to a computerized digital image scanning system of histomorphometry (Soft imaging Systems, Münster, Germany). To obtain the implant-bone contact percentage, an arithmetic mean was calculated for the sections of each implant. The arithmetic mean and the standard deviation for each of the six groups of surfaces were calculated.

Statistical methods

Due to high variability between the minipigs, a nonparametric statistical approach has been chosen. A Kruskal–Wallis Test was applied to analyse the effects of surface coating and time. Because of multiple comparisons, the significance level was adjusted according to the Bonferroni procedure.

Results

Loss rate

Two coll and two coll/CS/DC/TGF/BMP coated implants were lost in two pigs in the 5 week group. This accounts for a loss rate of 8.3% and might be due to micromotion in between the implant and the surrounding bone, caused by chewing.

The animals were sacrificed in groups of two within four consecutive weeks, starting 3 weeks after implant placement. Considering this, two implants of each surface state could be evaluated for each week of interest.

SRμCT

The results generated using SRμCT determined the amount of mineralized bone volume. The highest volume within the lower recess, compared to the region of reference was determined for the coll/CS coated implant (~84%). Implants coated with coll/CS/BMP and coll/DC/TGF followed with a slightly lower result (~80%). The coll coated implant showed (~75%). A much lower volume of mineralized bone was detected for the coll/DC implant coating (~20%) as well as for the coll/DC/CS/TGF/BMP coating (~17%).

Histomorphometry

At each week of observation, coll/CS or coll/CS/BMP showed the highest BIC of all surface states. This applies to the overall surface as well as to the recesses. For the total implant surface, BIC was statistically significantly increased for coll/CS and coll/CS/BMP at week five ($p = 0.030$) and six ($p = 0.025$) compared to the other coatings. The same applied to the recesses at week five ($p = 0.040$) and six ($p = 0.005$).

At the last week of observation, coll/CS/BMP reached the highest median BIC compared to the other surface coatings, measuring 46.3% for the overall implant surface. This was followed by coll/CS with 35.1% BIC. Observing the recesses at the last week of observation, coll/CS reached the highest median BIC of 57.9%, followed by coll/CS/BMP with 47.3% (Fig. 3, Tables 1 and 2)

Fig. 3 Histomorphometry: Histological section showing the bone-implant contact within the recesses; collagen/CS coated implant three weeks after placement (original magnification × 4, multiple alignment technique, Masson-Goldner)

Table 1 Percentage of bone-implant contact at the outer implant surface at 3, 4, 5 and 6 weeks after placement

Coating	Bone implant contact (BIC) (%) week				
	3	4	5	6	Total
Collagen	15.09	12.18	/	12.76	12.45
Collagen/decorin	14.77	7.19	3.24	12.65	12.65
Collagen/CS	**29.91**	**19.64**	**43.77**	*35.07*	**28.28**
Collagen/decorin/TGF	*19.13*	7.60	10.04	24.07	16.27
Collagen/CS/BMP	10.02	*16.75*	*17.52*	**46.32**	*22.92*
Collagen/decorin/CS/TGF/BMP	7.40	7.86	/	22.28	11.43

Bold indicates highest BIC per week; italics indicates second highest BIC per week

Table 2 Percentage of bone-implant contact within the recesses at 3, 4, 5 and 6 weeks after placement

Coating	Bone implant contact (BIC) (%) week				
	3	4	5	6	total
Collagen	6.54	0.00	/	8.70	1.81
Collagen/decorin	0.00	0.00	0.00	8.70	2.70
Collagen/CS	**12.38**	*15.12*	**39.06**	**57.90**	**22.05**
Collagen/decorin/TGF	*8.46*	10.01	7.98	17.78	12.63
Collagen/CS/BMP	2.91	**20.56**	*22.79*	*47.32*	*20.56*
Collagen/decorin/CS/TGF/BMP	0.81	2.07	/	12.63	2.40

Bold indicates highest BIC per week; italics indicates second highest BIC per week

Discussion

The aim of the present animal study was to investigate the effects of differently composed ECM implant coatings on osseointegration, both with and without the addition of recombinant growth factors.

Growth factors, especially the BMPs, can be highly effective in improving the healing process and in realizing an optimal osseointegration of endosseous implants. A large number of studies exist which demonstrate the ability of the BMPs to induce the formation of new bone [29–31]. This is of high interest especially in areas of poor periimplant bone quality. Still discussed is the fact that as a rule unphysiologically high amounts of recombinant BMP have to be applied to achieve the desired effect, often using collagen carriers.

Analysing histomorphometry, the inclusion of growth factors to surface coatings did not show the effect of inducing a statistically higher osteogenesis compared to the same surface lacking the growth factor. As in this case only a very low amount of growth factor was applied (400 ng/implant BMP-4 and 20 ng/implant TGF-β1) compared to the amount usually utilized in other studies [32–35]. This might be the reason for the very slight effect detected as opposed to the promising results reported in literature. Bessho used a mixture of 10 μg BMP and type I collagen in a 2 mL acidified saline solution [33]. Wikesjo describes a model where he applied 0.4. and 0.75 mg/mL BMP dilutions to increase osteogenesis [35].

The effects of the studied surfaces became apparent in the resulting differences in BIC. Bone implant contact at the outer implant surface was significantly increased for coll/CS and coll/CS/BMP at week five and six compared to the other coatings. The same applied to the recesses. The addition of the recombinant growth factor TGF-β1 to coll/DC coated implants did lead to an increase in BIC compared to a collagen/decorin coating. Still, the levels of BIC did not reach the levels of coll/CS or coll/CS/BMP coated implants.

Incorporation of both growth factors TGF-β1 and BMP-4 into a combined collagen/CS/decorin coating did not lead to an enhanced effect; if anything, the response appeared to decrease. This may be due to the fact that, even though BMP and TGF are known to act synergistically [22, 36], in vitro experiments showed the reaction to depend on the temporal pattern of the presentation, with a simultaneous application being detrimental [37]. Possibly this is also the case in the in vivo situation, and care should be taken to establish an appropriate release pattern if more than one growth factor is to be applied.

The most interesting result of this study was the fact that, of all surface states tested, the coating composed of

collagen and chondroitine sulphate without the addition of growth factors proved to be at least as good as with the inclusion of BMP-4. The data is statistically significant for the weeks five and six and favours the coll/CS surface. This could conceivably be occasioned by several effects above and beyond a direct interaction with adherent cells. If the matrix is not preloaded with recombinant growth factors, it is conceivable that endogeneous growth factors released at the implantation site can interact with the matrix and thus be stored for a certain time in the vicinity of the implant, which might be beneficial for healing. Another possibility is the interaction with inflammatory factors such as interleukins, which are reported to interact with glycosaminoglycans [38–40] and which can influence bone regeneration [41–43]. A potential interaction of a collagen/CS coating with inflammatory factors is also indicated by the fact that such composites give rise to a reduced inflammatory response [44].

The results of the SRμCT bone volume measurement have to be considered with care as only one sample per surface state was analysed. The results support the positive effect of CS, as the coll/CS coated implant showed the highest volume of mineralized bone. The addition of individual growth factors increased the bone volume over the reference collagen, while the simultaneously addition of both growth factors, BMP and TGF had a detrimental effect.

It can be concluded, that the osseointegration of titanium implants can be influenced in different degrees by modifying the surfaces using ECM components in combination with collagen. The data shows coll/CS and coll/CS/BMP coated implants to give rise to a higher degree of bone formation at the implant interface compared to collagen and the other surface modifications tested.

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