Improved Anchorage of Ti6Al4V Orthopaedic Bone Implants through Oligonucleotide Mediated Immobilization of BMP-2 in Osteoporotic Rats

Julia V. Wölfle1, Jörg Fiedler2, Lutz Dürsel3, Judith Reichert4, Dieter Scharnweber4, Anne Förster5, Bernd Schwenzer5, Heiko Reichel1, Anita Ignatius3, Rolf E. Brenner2*

1 Department of Orthopaedic Surgery, Centre of Musculoskeletal Research, University of Ulm, Ulm, Germany, 2 Division for Biochemistry of Joint and Connective Tissue Diseases, Department of Orthopaedic Surgery, Centre of Musculoskeletal Research, University of Ulm, Ulm, Germany, 3 Institute of Orthopaedic Research and Biomechanics, Centre of Musculoskeletal Research, University of Ulm, Ulm, Germany, 4 Max Bergmann Center of Biomaterials, TU Dresden, Dresden, Germany, 5 Institute of Biochemistry, TU Dresden, Dresden, Germany

Abstract

The aim of the present study was to test the biocompatibility and functionality of orthopaedic bone implants with immobilized oligonucleotides serving as anchor stands for rhBMP-2 and rhVEGF-A conjugated with complementary oligonucleotides in an osteoporotic rat model. Al2O3-blasted acid etched Ti6Al4V implants, carrying oligonucleotide anchor strands and hybridized with rhBMP-2 or rhVEGF-A through complementary 31-mer oligonucleotide stands were inserted into the proximal tibia of ovariectomized rats. At the time of surgery (15 weeks after ovariectomy) microCT analysis showed significantly lower bone mineral density compared to non-ovariectomized animals. Bone-implant contact (BIC) and pullout-force were not negatively affected by non-hybridized anchor strands. Twelve weeks after surgery, a significantly higher pullout force was found for BMP-2 hybridized to the anchor strands compared to non-hybridized anchor strands or native samples, and on histomorphometric analysis BIC was highest in the BMP group. Thus, we could show the biocompatibility and in vivo functionality of this modular, self-organizing system for immobilization and subsequent release of BMP-2 in vivo.

Introduction

The coincidence of osteoarthritis and osteoporosis represents a major challenge to total joint replacement in orthopaedic surgery: Makikinen et al. found 74% osteopenic or osteoporotic patients in a subgroup of 53 female patients with advanced primary hip osteoarthritis scheduled for cementless total hip arthroplasty [1]. Whereas the relationship between osteoporosis and failure rate of dental implants is controversially discussed in literature [2–8], studies on the impact of osteoporosis on osseointegration of orthopaedic implants are less numerous and much less controversial. In an osteoporotic animal model impaired implant osseointegration into the tibia or femur has been repeatedly seen [6,9], most recently by Stadlinger et al. [10]. Alm et al. [11] found bone loss in Gruen zone 7 after cementless total hip arthroplasty in female patients with low bone mineral density, and Aro et al. [12] recently reported that low bone mineral density negatively affects initial stability and delays stem osseointegration after cementless hip joint replacement in women. Orthopaedic implant anchorage primarily depends on osteoconduction from perimplant bone tissue. Therefore, roughness and physico-chemical properties of implant surfaces have been optimized to increase osteoconductivity [13]. A further approach to improve osseointegration of titanium implants in osteoporotic bone is surface modification with bioactive molecules encouraging bone formation. In this context the method of binding and/or release of such factors is a crucial issue [13]. Promising results have been published by Michael et al. who achieved immobilization of bioactive molecules on the implant surface in a two-step procedure: First, short strands of nucleic acids – “anchor strands” – were entrapped into a titanium oxide layer on the implant surface [14,15]. Then, conjugates of bioactive molecules and complementary strands were hybridized to the surface modified with anchor strands [14,16]. It has been shown that bioactive molecules thus fixed on a titanium surface retain their bioactivity: Conjugates of RGD peptides and complementary strands hybridized to anodic immobilized anchor strands increased osteoblast attachment on the titanium surface compared to the control group [16].

Promising bioactive molecules for surface modification are bone morphogenetic protein 2 (BMP-2) and vascular endothelial growth factor A (VEGF-A): BMP-2 is probably one of the most important growth factors in bone formation which is currently used in a wide range of tissue-engineering products that allow complete regeneration of bone e.g. in long bone defects or pseudarthrosis [17–19]. VEGF-A induces angiogenesis, which is necessary for blood supply and may also provide osteoprogenitor cells derived from multipotent pericytes for implant integration [20]. Moreover, it exerts a chemoattractive effect on human mesenchymal progenitor...
cells [21–24]. Previous in vitro studies using the oligonucleotide-mediated immobilization technology indicated that BMP-2 and VEGF-A could be effectively bound to titanium surfaces. The growth factors were released over an extended time span and induced stimulatory effects on proliferation and osteogenic differentiation of mesenchymal stem cells as well as proliferative effects on endothelial cells in vitro [25,26]. Biological activity of released oligonucleotide conjugated BMP-2 and VEGF-A was preserved as indicated by the induction of alkaline phosphatase in C2C12 cells or van Willebrand Factor in mesenchymal stem cells in vitro [25,26].

Since in vivo responses and functionalities have not been tested so far, the aims of this study were (i) to investigate the biocompatibility of the above-mentioned surface modification and (ii) to explore the effects of BMP-2 and VEGF-A functionalization on orthopaedic implant osseointegration in the osteoporotic bone. Therefore, Al2O3-blasted acid etched titanium (AAT) implants, with anchor strands (ODN), and with rhBMP-2 modified with 31-mer Oligonucleotides (BMP-ODN) and rhVEGF-A modified with 31-mer Oligonucleotides (VEGF-ODN) hybridized to the anchor strands were inserted into the tibia metaphysis of female rats 15 weeks after ovariectomy.

Materials and Methods

Ethics Statement

The animal trial was conducted according to relevant national and international guidelines; the study was approved by the local governmental authorities (Regierungspräsidium Tübingen, registration number 982). All surgery was performed under adequate anaesthesia and analgesia, and all efforts were made to minimize suffering.

Animals

Sixty-four female ovariectomized WISTAR rats were supplied by Charles River Laboratories (Killegg, Germany). Ovariectomy had been performed at the age of 10 weeks. They were kept under climate-controlled conditions (21 +/- 1.5°C, 47.5 +/- 7.5% humidity, light-dark-cycle 12 hours/12 hours). Access to phytoestrogen-free diet and tap water was ad libitum. Mean body weight at the time of surgery was 397 ± 32.2 grams.

Preparation of the orthopaedic bone implants

The orthopaedic implants consisted of an aluminium-oxide-blasted Ti6AlV4 cylindrical rod 5.2 mm in length with a diameter of 1.6 mm (kindly provided by Peter Brähm GmbH, Weisendorf, Germany). One end of the rod was threaded (length 1.2 mm) to enable biomechanical pullout testing. The samples were acid etched for 120 s in a mixture of 0.4 M HF and 1 M HNO3 at room temperature and following cleaned two times 15 min in ultrapure water in a ultrasonic bath. Surface structure of the implants before and after acid etching is shown in Figure 1. Briefly, anodic polarization of the implants was performed in a custom electrochemical cell from acrylic glass with a gold wire acting as counter electrode and an Ag/AgCl reference electrode connected to the cell via a buffer-agarose salt bridge. Polarization was performed with the electrochemical system Voltalab 4.0 combined with a high-voltage booster HVB100 (Radiometer Analytical, Copenhagen). Ethanolic acetate buffer from 0.5 M acetate containing 5 M ethanol, pH 4.0 and phosphorylated 60mer single stranded oligonucleotides – briefly called anchor strand (AS) - at a concentration of 400 nM was used as electrolyte. Galvanostatic polarization at 7 mA/cm² was performed until a potential of 14.5 VAg/AgCl was reached. After several desorption steps (three times in the ethanolic acetate buffer, twice in sterilized ultrapure water; each for 30 s) to remove merely adsorbed and not entrapped anchor strands the implants were packed under dry argon atmosphere and sterilized by gamma irradiation with a standard dose of 25 kGy [14,16]. Conjugates of complementary strands of nucleic acid and recombinant human rhBMP-2 (Relatech, Braunschweig, Germany) or recombinant human rhVEGF-A (VEGF165, Relatech) respectively were hybridized to the surface modified with anchor strands directly before implantation under sterile conditions [25,26]. A final washing step was performed to eliminate unbound growth factors. Calculated from the density of single stranded oligonucleotides about 2 ng rhBMP2/mm² or 2 ng rhVEGF/mm² were immobilized on the orthopaedic implant surface.

Experimental design

The animals were randomly assigned 16 animals each to one of the following groups: (1) control group, (2) anchor strand group, (3) BMP-ODN group, or (4) VEGF-ODN group. According to which group they belonged to one of the following orthopaedic implants was inserted: (1) aluminium-oxide-blasted acid etched Ti6AlHV (AAT), (2) AAT with anchor strands (ODN), with (3) hybridized
To confirm reduced bone mineral density of the ovariectomized rat, four additional animals were sacrificed fifteen weeks after ovariectomy – i.e. at the time of surgery – and the bone mineral density of their fourth lumbar vertebral body was compared to that of six otherwise identical animals who had not undergone ovariectomy.

**Surgical Procedure**

Fifteen weeks after ovariectomy the above-mentioned implants were inserted into the proximal tibia on both sides. Anaesthesia was administered by means of an inhalation device (Isolfrane 2%) and subcutaneous injection of analgesics (Tramadolor 20 mg/kg). The surgical technique was similar to the one described by Dayer et al. [27]: A 10 mm incision was made at the medial aspect of the proximal tibia, and the periosteum was incised ventrally to the medial collateral ligament. A 1.7 mm drill hole was made level with the insertion of the patella tendon just ventrally to the medial collateral ligament using hand-held drills held strictly perpendicular to the longitudinal axis of the tibia. The orthopaedic implant was then inserted into the bone. The threaded part remained outside and was covered by a 2 mm tube cut off from a venous catheter (fluorinated ethylene propylene, Vasofixx Braun® 18 G, Braun B., Melsungen, Germany) in order to prevent osseous overgrowth. Postoperative analgesia was ensured by adding Tramadolor to the drinking water (25 milligram per litre). Antibiotics (Clindamycine 45 mg/kg) were administered subcutaneously daily on the first three postoperative days.

**Biomechanical Testing**

For biomechanical testing a specifically designed cylindrical device with a matching internal thread was screwed onto the threaded part of the orthopaedic implant of the left tibia. To determine the pullout force of the implant the device was attached to a 200 N load cell (HBM, Darmstadt) of a standard testing machine (Z010, Zwick, Ulm, Germany; see Figure 2A). A force-displacement diagram (test speed 10 mm/s, preload 0.5 N) was recorded by the testing software (testXpert II, Zwick, Ulm, Germany) and the load occurring before the first sudden drop of the tensile force was defined as maximum pullout force (see Figure 2B).

**Histomorphometric measurement**

The right tibia including the inserted orthopaedic implant was embedded in Technovit VLC7200 (Kulzer, Germany) and ground down to sections of 100 μm along the longitudinal axis of the tibia. Masson-Goldner staining of the sections was used to visualize connective tissue surrounding the implant. The sections were inspected and scanned with a fully automated inverted light microscope (Leica DMI6000B, Wetzlar, Germany). To quantify the amount of bone surrounding the implant the following parameters were determined semi-automatically with the aid of an imaging analysis software (MetaMorph®, Leica, Wetzlar, Germany): The bone-to-implant contact rate was calculated by dividing the total length of bone-to-implant contact by the total length around the orthopaedic implant within the tibia. The bone density within the medullar cavity was defined as the percentage of the area of osseous tissue within a 1 mm strip of medullar cavity just distal to the orthopaedic implant.

**MicroCT scanning**

The fourth vertebral body of the lumbar spine as well as two densitometric phantoms (250 mg/cm³ and 750 mg/cm³ hydroxyapatite bone-equivalent density) were scanned with a microCT.
system (Skyscan 1172, Kontich, Belgium) with a resolution of 10 µm. As region of interest the trabecular bone starting ten slides below the upper endplate and ending ten slides above the lower endplate was then manually selected. After calibrating the image processing software (CT Analyser V1.11.4.2, Skyscan 1172, Kontich, Belgium) with the assistance of the two-densitometric phantoms the bone mineral density of the region of interest was determined. Then, the grayscale image was reduced to a binary image using Otsu’s method of histogram shape-based image thresholding. Five bone structural parameters (bone volume fraction BV/TV, connectivity density Conn.D (measuring the degree of connectivity of trabeculae normalized by TV as explained in Bouxsein et al. [28]), trabecular number Tb.N, trabecular thickness Tb.Th, and trabecular separation Tb.Sp) were then automatically determined from the region of interest (CT Analyser V1.11.4.2, Skyscan 1172, Kontich, Belgium).

Statistical analysis

For statistical analysis the Statistical Package for Social Sciences (SPSS® Inc., IBM, version 19) was used. Due to the low number of animals per group non-parametric tests were used for statistical analysis. Mann-Whitney-U-Test was used to compare two groups (e.g. bone mineral density), Kruskal-Wallis-Test for more than two groups (e.g. pullout force). A probability value of less than 0.05 was considered to indicate statistical significance. Boxplots and median values were used to characterize the distribution of continuous variables.

Results

MicroCT scanning

MicroCT analysis of the four additional animals sacrificed 15 weeks after ovariectomy – i.e. at the time of the surgery – showed significantly lower bone mineral density when compared to six animals who had not undergone ovariectomy. More specific data is depicted in the supplement (Table S1).

Histomorphometric measurement

Within the sections of the orthopaedic bone implant inserted into the tibia a thin layer of osseous tissue covered large parts of the implant. The remaining surface of the orthopaedic implant was covered to a certain extent with a thin layer of non-mineralized connective tissue. This is exemplarily shown in Figure 3 by Masson-Goldner-Stains of non-decalcified 100 µm sections of the orthopaedic implants and the surrounding osseous tissue. Four weeks after surgery no relevant difference of the bone-to-implant contact rate (BIC) of the orthopaedic implants of all four groups was measureable (p = 0.927, see Fig. 4). Twelve weeks after surgery bone-to-implant contact was highest in the BMP group (median 60.7%) though no significant difference between the groups could be found (p = 0.927).

In all ovariectomized animals cancellous bone was scarcely observed within the medullar cavity. Four weeks after surgery we did not see a significant difference between the four treatment groups (p = 0.841). With exception of the BMP-ODN group we found a further decrease of the bone density in the animals...
sacrificed after 12 weeks when compared to the animals sacrificed after 4 weeks. As shown in Fig. 5, 12 weeks after surgery bone density was highest in the BMP-ODN group (median BD 14.3%) though we did not find a significant difference between the four groups (p = 0.488).

**Biomechanical testing**

In the animals sacrificed after four weeks maximum pullout force was not significantly different between the treatment groups. As presented in Figure 6, we found a significant difference (p = 0.034) 12 weeks after surgery. Pullout force was significantly higher in the BMP-ODN group (median 81.8 N) in comparison with both the AAT group (median 57.0 N) as well as the ODN group (median 65.0 N).

**Discussion**

This animal study represents the first *in vivo* investigation of a new kind of orthopaedic implant surface functionalization, based on bioactive molecules immobilized through short complementary oligonucleotide anchor strands on a titanium surface using the specificity of nucleic acid hybridisation.

For reasons of clinical relevance ovariectomized rats were chosen for the study to simulate the compromised bone stock found in osteoporotic bone. The ovariectomized rat has been used as a model for osteoporosis in numerous studies [29-33]. It has been shown that after ovariectomy a biphasic pattern of cancellous bone loss occurs: During the first 100 days there is a rapid bone loss similar to high-turnover osteoporosis, which characterizes postmenopausal osteoporosis. Afterwards there is a relative stabilization at an osteopenic level with a slow bone loss resembling low-turnover osteoporosis, which is characteristic for senile osteoporosis [32,34]. Okamura et al. found that this
difference of bone turnover in the ovariectomized rat affects the
bone formation around a titanium implant: There was a
significantly lower bone contact rate of the implant in the animals
with low bone turnover (i.e. 15 weeks after ovariectomy) when
compared to the high bone turnover group (i.e. 3 weeks after
ovariectomy) [31]. Since we were focusing on senile osteoporosis,
we performed the orthopaedic implant insertion 15 weeks after
ovariectomy, i.e. in the phase of low-turnover osteoporosis. The
decrease in bone density of the ovariectomized rats was confirmed
by local histomorphometric and humar microCT analysis. This is
in agreement with previous data from ovariectomized rats
indicating the development of osteoporosis in both the spine and
proximal tibia in the respective age and time span after
ovariectomy [35].

Our results confirm the biocompatibility of the novel surface
modification: no inflammatory response surrounding the orthopa-
decum implant surface by hybridisation to short strands of
nucleic acid used in both cases (5 double layers of polyanionic
dNA ± 300 bp/molecule vs. a single layer of 60mer ODN). With
respect to biocompatibility our findings correspond with their
findings because they neither described any inflammatory cells
surrounding the implant or differences of the tissue response
between the coated and uncoated implants [36]. Moreover, we did
not observe any case of infection indicating that the sterilization by
gamma irradiation before the hybridisation step was effective.

Additionally, our data suggest that BMP-2 immobilized on the
orthopaedic implant surface by hybridisation to short strands of
nucleic acids preserved its bioactivity in vivo and exerted a biologic
effect on the surrounding tissue. This corresponds to the recently
published in vitro study by Schlepke et al. [26]. They found a
retarded release of rhBMP-2 using this system of nanoanchoring
compared to a simple adsorption based strategy. In the respective
study only 20% of the hybridized conjugated BMP-2 had been
released within 4 weeks and the released conjugated BMP-2 was
biologically active as indicated by similar stimulation of alkaline
phosphatase in C2C12 cells compared to native BMP-2 [26]. If
the release kinetic was similar under in vivo conditions a sufficient
amount of bioactive BMP-2 would have been locally available in the
time span after surgery during which the benefit for
orthopaedic implant anchorage was observed. This in vivo
response also is concordant with previous in vitro results on
stimulatory effects of titanium surfaces carrying identically
immobilized BMP-2 on proliferation and osteogenic differentiation
of human bone marrow stroma cells [26]. Accordingly, in the
animals of the BMP-ODN group sacrificed after 12 weeks we
found a significantly higher pullout force when compared to both
the AAT and ODN group. Similarly, the median bone-to-implant
contact rate and median periimplant bone density were highest in the
BMP-ODN group 12 weeks after surgery. A number of
previous animal studies using different species, dosages and
immobilization/carrier systems have demonstrated positive effects
of BMP-2 functionalization on primary stabilization of titanium
implants [37]. In these approaches BMP-2 is released early after
implantation and non-physiologically high concentrations have
been used [38]. With the oligonucleotide mediated immobilization
strategy we could observe positive effects on orthopaedic implant
anchorage with rather low concentrations of BMP-2 after the
primary phase of osseointegration. This may be explained by a
favourable balance of growth factor bound on the surface and the
amounts of BMP-2 available in diffusible form for an extended
time span. To our knowledge, comparable studies in estrogen-
deficient animal models of osteoporosis do not exist so far. In a rat
bone defect model of the femur, however, a positive effect of BMP-
2 loaded collagen carrier has previously been described which is
consistent with our results [39]. Moreover, for statin coating of
titanium implants with chitosan as a carrier positive effects on
bone implant contact after 2 weeks in ovariectomized rats have
recently been reported [10]. Statin induces the expression of BMP-
2 in osteoblastic cells and also has positive early effects on
osseointegration of implants in the non-compromised situation
[40].

Since a reduction of local blood supply and VEGF-expression
has been previously reported in ovariectomized mice [30] positive
results of the VEGF-hybridisation could be expected at the start of
our study. Nevertheless, we did not observe significant effects on
both bone-implant contact and pullout force up to 12 weeks after
implantation although an extended in vitro release of the growth
factor and in vitro bioactivity of the released conjugated VEGF-A
could previously be shown [25]. This may be partly explained by
recent data of Lui et al. demonstrating that intracellular effects of
VEGF-A but not the extracellular interaction with membrane-
bound receptors are responsible for induction of osteogenic
differentiation of mesenchymal stem cells [41]. Since the outcome
parameters used in our study depend on the induction of
mineralized bone tissue, we might have missed associated effects
on vascularization or recruitment of undifferentiated mesenchymal
stromal cells. We did not observe major differences in blood vessel
density around the subcutaneous implants on a macroscopic level.
Therefore, we cannot exclude that the local concentrations of
released conjugated VEGF-A were too low to exert a biologic
response. On all things considered, the question of a possible
benefit of VEGF-A for orthopaedic implant integration in
estrogen-deficient osteoporotic animals clearly deserves further
investigation. Published in vivo studies addressing this issue are not
available so far. If an effect on vascularisation could be achieved, a
combined BMP-2- and VEGF-hybridisation might have the
potential for further optimization of orthopaedic implant anchor-
age in osteoporotic bone.

Conclusion

Our study is the first in vivo trial that shows the biocompatibility
of an orthopaedic implant surface modification with short single
strands of nucleic acids serving as anchor strands for complemen-
tary oligonucleotide conjugated bioactive molecules. Entrapped
into a superficial titanium oxide layer of a Ti6Al4V implant the
system is able to preserve the bioactivity of BMP-2 in vivo.
Orthopaedic implant osseointegration in the compromised bone
stock of estrogen-deficient osteoporotic rats was enhanced by
hybridized conjugated BMP-2 molecules in a later phase of
implant anchorage between 4 and 12 weeks after surgery
compared with the native commercially used aluminium oxide-
blasted and acid etched surface. This novel technique to
functionalize biomaterial surfaces combines stable attachment of
bioactive molecules and modifiable release kinetics with a high
degree of flexibility in the production process. Therefore, its
application for total joint replacement in osteoporotic patients
seems promising.
Supporting Information

Table S1

Acknowledgments

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