

Coating of titanium implants with type-I collagen

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Abstract

Purpose: Type-I collagen, the major structural protein in bone, has beneficial properties regarding bone regeneration. Little is known about the potential effects of collagen coating on orthopedic implants.

Methods: 3 to 6 $\mu\text{g}/\text{cm}^2$ of lyophilized type-I collagen was absorbed on titanium rods. Six coated and uncoated pins of 0.9 mm diameter were inserted into the tibia of adult male Wistar rats for 1, 2, 4, 7, 14, and 28 days. Specimens were embedded in methacrylate-based Technovit 9100N resin. From one portion cutting and grinding sections were obtained. The implant was removed from the other half that was depolymerized, sectioned, and mounted for immunohistochemistry.

Results: At day 4, the interface around the collagen-coated implants displayed a granulation tissue with higher numbers of cathepsin D-positive mononucleated cells compared to the uncoated implants ($p < 0.05$). Active osteoblasts, reactive for osteopontin, were increased around the collagen-coated pins at day 4 and 7 ($p < 0.01$). After 28 days of implantation, direct bone contact averaged 74.9% around the collagen-coated implants and 62.1% around uncoated implants (NS). The amount of newly formed bone averaged 76.3% around the collagen-coated pins and 67.8% around the uncoated pins (NS). The histomorphometric findings were confirmed by SR μ CT in two specimens.

Conclusions: The earlier observation of mononuclear phagocytosing cells and the earlier and higher expression of bone-specific matrix proteins suggest an increased early bone remodeling around titanium pins through collagen coating. A tendency towards increased bone formation was observed around the coated implants.

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Introduction

Extensive research has focused on hydroxyapatite (HA) coating of orthopedic implants to improve the mechanical strength of bone bonding. While positive properties of HA-coated prostheses [2,9,28] and external fixation devices [4,23] have been reported, several potential drawbacks exist like inhibition of osteoblasts in vitro [43] and induction of foreign body reactions by HA particles, which may encapsulate in the soft tissues [24,40]. The present study aims at improving the biocompatibility of titanium implants by coating the implant surface with type-I collagen, the major structural

protein in bone. While collagen has been used with favorable results in several surgical and orthopedic applications [7,8,19], we are not aware of any in vivo study on coating of metallic implants with type-I collagen for potential use on orthopedic implants.

The host response to surgical implants is mediated by regulatory interactions between the cells and the extracellular matrix [30]. Several cell-surface receptors for extracellular matrix molecules are present on cells in the interface region like osteoblasts, osteoclasts, fibroblasts, macrophages, and multinuclear giant cells, regulating the host response to surgical implants and tissue regeneration [47]. The present study was designed to describe these early processes taking place in the interface region with special focus on cell–matrix interaction. Therefore, we characterized the immigrated and orthotopic cells around the implant and their extracellular matrix products delivered into the interface region

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between bone and implant with immunohistochemical methods.

Immunohistochemical characterization of the bone–implant interface on metallic implants embedded into methyl-methacrylate-based media is technically demanding [44]. For immunohistochemical staining, specimens are depolymerized, decalcified and re-embedded in paraffin [13]. Some embedding media display an exothermic reaction during polymerization with potential damage to the tissue and loss of antigenicity [44]. This study was designed to evaluate the bone implant interface of titanium pins in the rat tibia with both histological and histomorphometrical methods on undecalcified cutting and grinding bone sections after cold polymerization with the implant in situ and immunohistochemical characterization of the interface after removal of the implant, depolymerization, and decalcification. In a pilot study, histomorphometric results were compared with that of microtomographic scanning.

Materials and methods

Preparation of the pins

Lyophilized type-I collagen from calf skin (Fluka, Heidelberg, Germany) was used for fibrillogenesis at 37 °C for 4 h, centrifuged twice, and absorbed for 15 min on 0.9 mm pure titanium rods. After irrigating with distilled water and drying, 3–6 µg/cm² of type-I collagen was absorbed (Fig. 1). Uncoated pins of pure titanium served as controls. All pins were sterilized with ethylene-dioxide.

Operative technique

Permission for the experiments was granted by the local animal care committee. Seventy-two adult male Wistar rats (Charles River Laboratories, Sulzfeld, Germany) with a mean weight of 400 g were used for the study. Titanium Kirschner wires of 0.9 mm diameter, either uncoated or coated with type-I collagen, were inserted into the tibia of the animals in the fashion of intramedullary nails. After initial sedation with mixed O₂/CO₂ inhalation, the animals received intramuscular injectional anesthesia with Xylazine (0.2 mg/kg) and Ketamine (10 mg/kg). Apart from the injection the animals experienced no pain throughout the experiments. After hindlimb shaving and disin-

fection, the pins were introduced through stab incisions via the tibial tuberosity into the medullary canal of the tibia. The pins were cut at the bone surface and the skin incisions were closed with absorbable sutures. Post-operatively, the animals received analgesics for 48 h (Tramadol) within the drinking water and were allowed to move freely without restriction of weight-bearing. The respective groups of 6 animals with coated and uncoated pins were sacrificed with carbon dioxide inhalation after mixed O₂/CO₂ inhalation at days 1, 2, 4, 7, 14, and 28 post-implantation. The hindlimbs were disarticulated at the knee joint and the tibiae were freed completely from all adherent soft tissue. The fibulae were removed.

Histological preparation

After immediate fixation in 1.6% paraformaldehyde for 24 h at 4 °C, the specimens were immersed in 0.02 M phosphate buffer with 5% sucrose at pH 7.4, freshly prepared from cold stock solutions (1 part 8% paraformaldehyde solution, 1 part 0.04 M phosphate buffer and 10% sucrose solution at pH 7.4 mixed with 2 parts double distilled water). After fixation, tissues were washed overnight at 4 °C in 0.02 M phosphate buffer with 5% sucrose pH 7.4. Specimens were dehydrated with ethanol in increasing concentrations. After dehydration the samples were impregnated with equal parts of stabilized methyl-methacrylate (MMA) and xylene for 1 h at 4 °C. For the second step of immersion a mixture of unstabilized MMA, *n*-butyl-methacrylate (BMA), and polyethylene glycol 400 (in a ratio of 40:60:2-parts) was used containing 1.5% dried benzoyl peroxide (BPO) as a catalyst.

For polymerization each specimen was transferred into a gelatine capsule, filled with a fresh mixture of 2 units stock embedding medium A and 3 units stock embedding medium B, capped, and stored at –20 °C. Polymerization was completed within 24 h. The polymerized blocks were allowed to warm up to 4 °C over 2 h and stored at room temperature (embedding media A and B provided in the Technovit 9100N[®] kit, Heraeus-Kulzer, Friedrichsdorf, Germany) [44]. The hardened specimen blocks were cut horizontally by sawing with Exact[®] cutting and grinding equipment (Exact, Norderstedt, Germany). From the diaphyseal portion of the tibia horizontal cutting and grinding sections of approximately 15 µm were obtained and stained with hematoxylin and eosin (H&E) and Goldner-Masson trichrome. The metaphyseal portion of the specimen block was cut longitudinally through the implant. The titanium pin was then carefully removed and specimens were cut horizontally in serial sections of 5 µm with a hard blade knife (Leica, Bensheim, Germany). The sections were mounted on silane-coated slides and dried for 48 h at 37 °C.

Immunohistochemistry

For immunohistochemical staining the sections were deacrylated three times for 2 h with methoxy-ethyl acetate (Merck Biomaterials, Darmstadt, Germany), and three times for 20 min with xylene. Then,

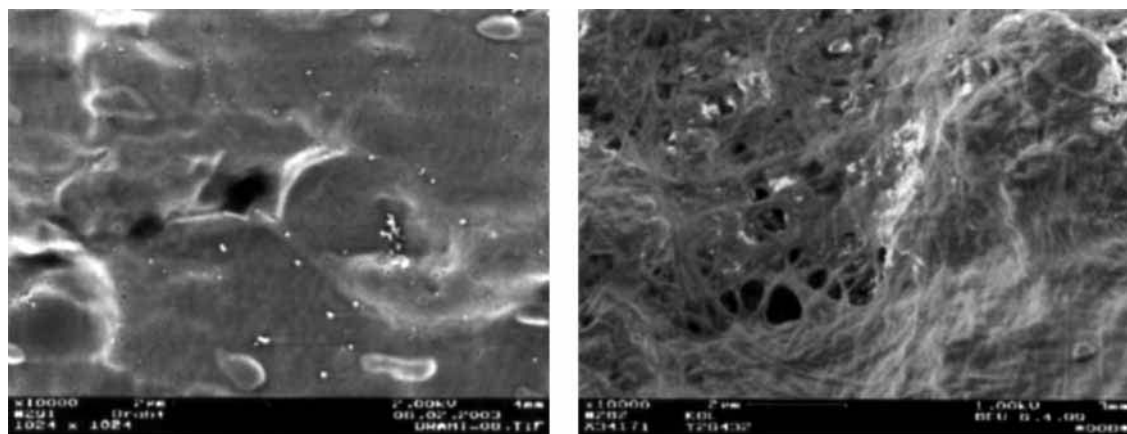


Fig. 1. Scanning electron micrograph of the uncoated (left) and collagen-coated (right) titanium pins (magnification 1:20,000).

the sections were rehydrated and irradiated twice for 5 min at 850 W with microwaves in 0.01 M sodium citrate buffer (pH 6.0). After washing in phosphate buffered saline solution (pH 7.4), the sections were treated with 0.3% hydrogen peroxide for 30 min, incubated with respective normal sera and then incubated for 1 h at 37 °C with primary antibodies (Table 1). The antibodies were detected with biotinylated secondary antibodies followed by a streptavidin/biotin-peroxidase complex (Vectastain Elite, Vector, Burlingame, CA). The peroxidase activity was visualized with 3'-3'-diaminobenzidine. Control procedures without primary antibodies were performed.

Histomorphometry

Histomorphometrical analysis was carried out with the Quantimed® software (Leica, Bensheim, Germany) on an IBM-compatible computer connected to a Leica microscope. Horizontal cutting and grinding sections of 15 µm thickness of six specimens of each group (uncoated and collagen-coated) retrieved after 28 days were stained with Goldner trichrome. After detection of bone and exclusion of bone marrow and fibrous tissue, the direct contact area between bone and the titanium implant was detected and expressed as a percentage of the total implant circumference. The amount of newly formed bone in the interface was calculated by measuring the total bone content within a circle of 0.1 mm around the implant.

Statistical analysis

Cathepsin D and ED 1-reactive mononucleated macrophages and multinucleated giant cells and osteonectin positive cells, located in the interface region between the implant and the orthotopic cancellous bone, were counted in two low-power fields of three subsequent histological slices per animal. Statistical analysis was performed with SPSS for Windows® (SPSS Inc. Chicago, IL, USA) using the Mann-Whitney *u*-test and Wilcoxon test. Statistical significance was assumed at $p < 0.05$. The differences of the average histomorphometric values (direct bone contact, bone content around implant) were evaluated with Student's *t*-test, significance level was set at $p < 0.05$.

Synchrotron microcomputed tomographic scanning (SRµCT)

Two embedded implants (one coated with type-I collagen and one uncoated) retrieved at 28 days post-implantation prior to cutting and grinding were cut into cylindrical forms of 4 mm in diameter and 10 mm in length, centered on the implant. The non-destructive investigations were performed at the Hamburg Synchrotron Laboratory (HASYLAB) of the Deutsches Elektronen-Synchrotron (DESY) at beamline BW2 with parallel monoenergetic X-rays of 26 keV photonic energy. The different X-ray absorption of titanium, bone and the embedding material (methyl-methacrylate) was scanned in 720 projections with an image size of 1536 × 1536 pixels and a pixel resolution of approximately 4 µm. With the aid of a filtered backprojection algorithm data from these scans were processed for three-dimensional reconstruction with an effective volume of 1535 × 1536 × 1024 pixels and a contrast resolution of approximately 12 µm. Three-dimensional visualization of the filtered absorption values was obtained with VGStudioMAX 1.1 software (VolumeGraphics, Heidelberg, Ger-

many). Mineralized bone appeared gray, titanium appeared white and the data for methyl-methacrylate were set transparent.

Results

Histological and immunohistochemical analysis

At the first day after implantation non-specific inflammatory reaction (migration of granulocytes and lymphocytes) was seen around both types of implants without apparent differences. At day 2, early granulation tissue with a large number of fibroblast-like cells, fibroblasts, and a small number of ED 1- and cathepsin D-positive mononuclear phagocytosing cells were seen around both coated and uncoated implants. No clusters of foreign body cells, like multinucleated giant cells, were observed around any implant.

Four days after implantation, at the interface region around the uncoated pins a primitive fibrin network with low infiltration of mainly granulocytes, fibroblasts and some macrophages, staining positively for ED 1 and cathepsin D, was observed (Fig. 2). At the same time, around the type-I collagen-coated implants a reparative granulation tissue was seen with a high amount of infiltration of fibroblasts, staining positively for Vimentin, and mononuclear macrophages displaying immunoreactivity against ED 1 and cathepsin D (Fig. 2). Many cells of the first layer at the implant surface showed the typical appearance of slender cells (Figs. 2 and 3). At days 4 and 7, osteogenic activity was detected directly at the interface with activated osteoblasts reactive against osteopontin around the collagen-coated implants only (Fig. 3). Morphologically the high osteogenic activity was reflected by formation of islands of woven bone within the fibrous interface from the 7th post-operative day on around the coated implants (Fig. 3). In contrast, osteopontin reactivity around the uncoated implants at days 4 and 7 after implantation, was confined to the local cancellous bone trabeculae, no such activity was detectable at the interface (Fig. 3). The same difference between coated and uncoated pins was observed for the staining against osteocalcin (not shown). Cells of mesenchymal origin, staining against vimentin,

Table 1
List of antibodies used for immunohistochemical analysis

Antibody	Type	Clone	Company
Cathepsin D	Polyclonal	–	BioGenex (San Ramon, CA, USA)
CD 3	Monoclonal	PS-1	Novo Castra (Newcastle, UK)
CD 8	Monoclonal	OX-8	PharMingen (Heidelberg, Germany)
CD 44	Monoclonal	OX-49	PharMingen (Heidelberg, Germany)
ED 1	Monoclonal	ED 1	Serotec (Oxford, UK)
Osteocalcin	Monoclonal	OCG-3	Zytotec (Berlin, Germany)
Osteopontin	Polyclonal	–	Chemicon (Temuco, CA, USA)
Vimentin	Monoclonal	V9	Dako (Glostrup, Denmark)

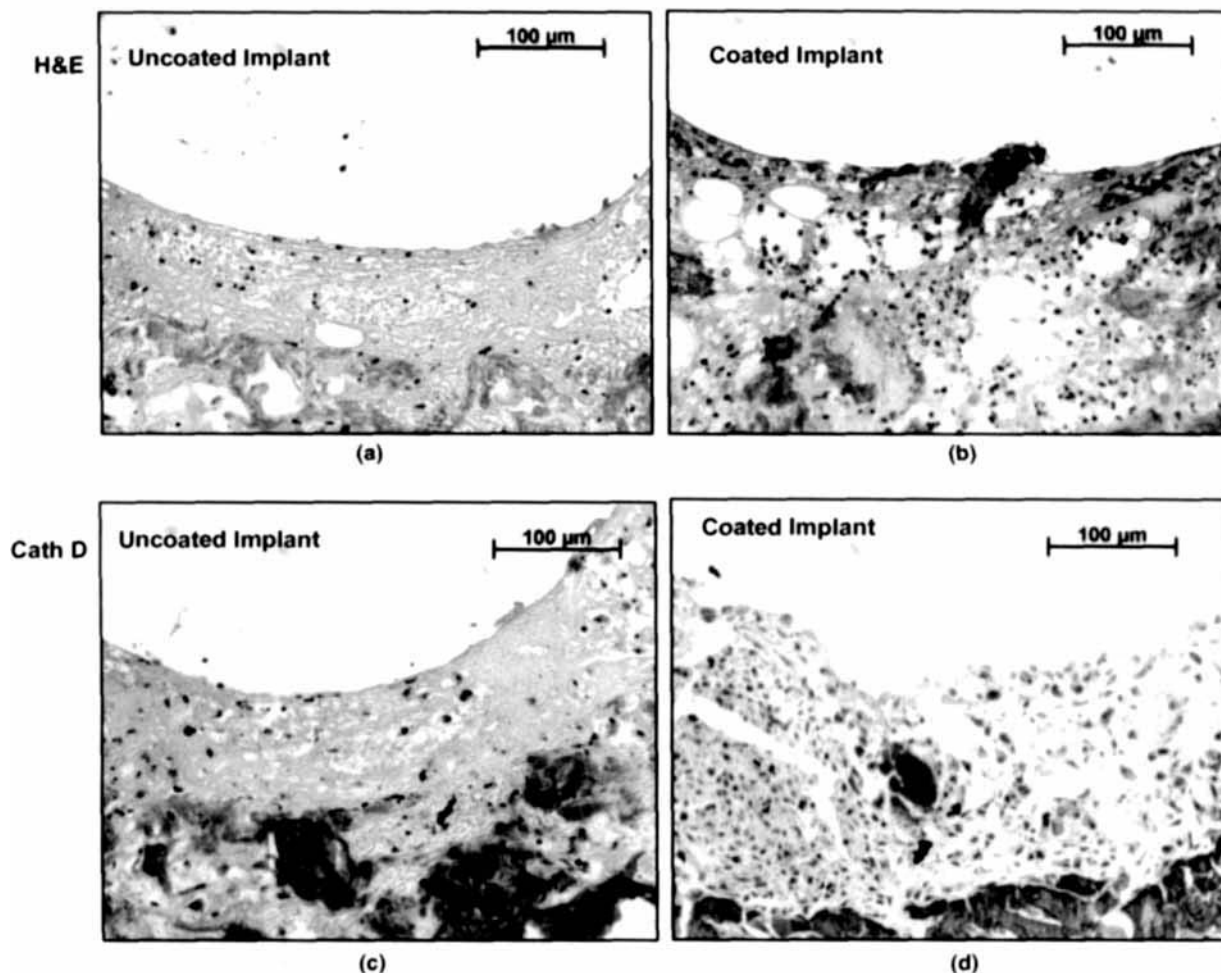


Fig. 2. Undecalcified sections of the bone–implant interface after removal of the pins at day 4 after implantation (original magnification 200 $\times$). H&E staining shows a primitive fibrin network around the uncoated pins (a), while the collagen-coated pins were surrounded by a granulation tissue rich in mononuclear cells (b). Immunoreactivity for cathepsin D (brown) was stronger around the coated (d) than the uncoated implants (c). (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

were seen around both types of implants, while vimentin activity appeared higher around the coated implants. These cells consisted mainly of fibroblasts (including slender cells) while very few lymphoid or endothelial cells were seen at these stages. However the differences between the groups were difficult to quantify because of the intra- and extracellular expression.

At day 14 after implantation a richly vascularized reparative fibrous tissue was observed around both uncoated and collagen-coated implants. Slender cells of both the osteoblast and fibroblast type were seen around both implants. Reactivity for both cathepsin D and ED 1 was increased around uncoated pins, while there were no differences with respect to vimentin, osteopontin, osteocalcin, or osteonectin at that time.

By day 28 after implantation both uncoated and type-I collagen-coated titanium pins were surrounded almost completely by newly formed lamellar bone with small parts of dense fibrous tissue connecting the distinct bone lamellae (Fig. 4a and b). This was observed both in the

metaphyseal and the diaphyseal parts of the tibia. The bone was in close contact with the implants without intervening fibrous tissue. In higher magnification images a small osteoid layer was seen between the newly formed bone and the implant (Fig. 4c and d). Between the two types of implants no apparent differences were noted with respect to new bone formation. No inflammatory response or adverse reaction was seen around either implant. No signs of bone resorption, foreign body reaction, or encapsulation of the implants were seen. Bone contact and the amount of new bone formation were evaluated quantitatively with histomorphometrical analysis (see below).

Statistical analysis of immunohistochemical markers

The total number of cathepsin-D- and ED 1-positive cells was higher around collagen-coated pins at day 4. While differences for cathepsin D reached statistical significance ($p < 0.05$), with the numbers available, no

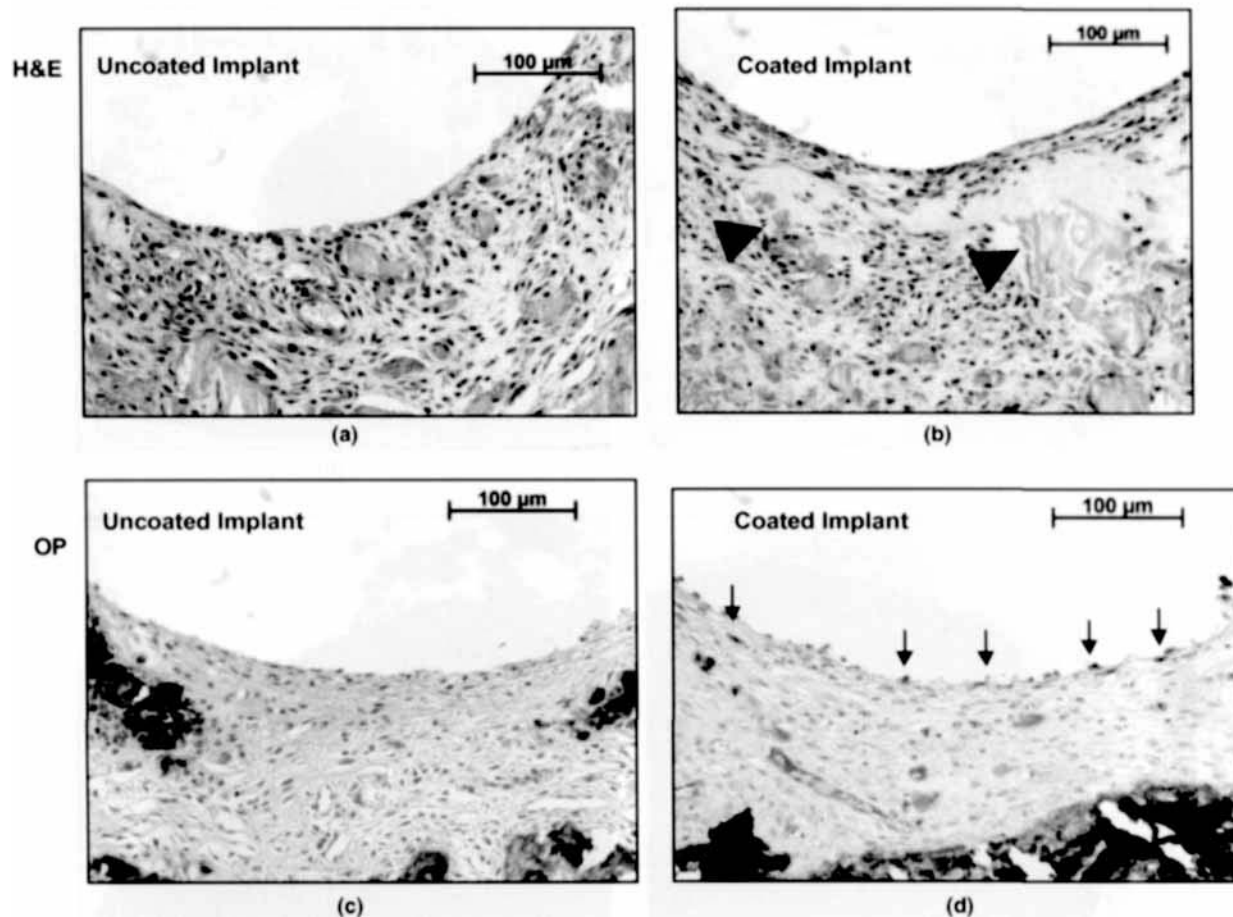


Fig. 3. Undecalcified sections of the bone-implant interface at day 7 after implantation (original magnification 200 $\times$). H&E staining shows a granulation tissue with newly formed capillaries around the uncoated pins (a). Small islands of woven bone (arrowheads) were observed already around the collagen-coated pins (b). The osteoblast marker osteopontin was observed within the local cancellous bone but not at the interface around the uncoated implants (c), while numerous active osteoblasts reactive against osteopontin (arrows) were seen directly at the interface around the collagen-coated implants (d).

statistically significant differences could be found for the ED 1-positive cells ($p = 0.07$). At 4 and 7 days after implantation significantly more osteocalcin positive cells were seen around the collagen-coated implants as compared to the uncoated implants ($p < 0.01$). At day 14 after implantation, the numbers of both cathepsin D and ED 1-positive cells were significantly higher around the uncoated implants ($p < 0.05$). At day 28, no significant differences between the two groups could be detected for any of the immunohistochemical markers (Fig. 5).

Histomorphometry

At 28 days the total bone implant contact averaged 62.1% (range 43.5–77.7%, SD 16.5) of the total circumference of the uncoated titanium implants and 74.9% (range 64.2–87.0%, SD 9.0) of the total circumference of the collagen-coated implants. With those numbers available, this difference did not reach statis-

tical significance ($p = 0.14$). The percentage of bone content within 0.1 mm around the pins averaged 67.8% (range 52.6–79.1%, SD 15.7) around the uncoated implants and 76.3% (range 57.2–86.3%, SD 11.3) around the collagen-coated implants. With the numbers available, no statistically significant differences were found with respect to the total amount of newly formed bone ($p = 0.31$).

Microtomographic (SR μ CT) findings

The filtered three-dimensional reconstructions (Fig. 6) obtained from the SR μ CT measurements show a clear contrast between the absorption values of mineralized bone and the titanium implant. Image artifacts close to the highly absorbing titanium were not seen. In analogy to the histomorphometrical measurements the mineralized bone contact to the implant surface was retrieved from the μ CT data. The mean bone-implant contact as a percentage of the total implant diameter was 51.0% for

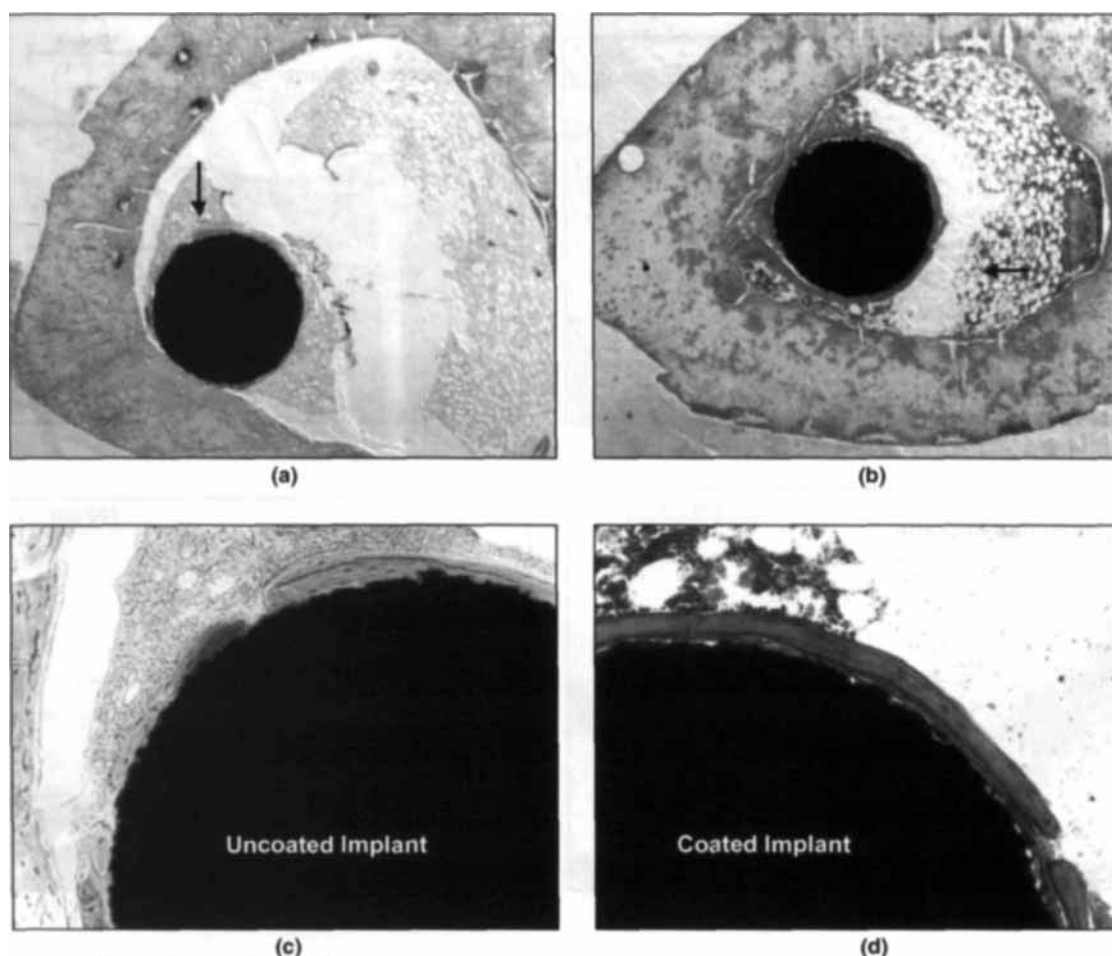


Fig. 4. Cutting and grinding sections of the uncoated (a) and collagen-coated implants (b) in the diaphyseal portion of the tibia at day 28 after implantation (H&E, original magnification 50 $\times$). Slightly more direct bone contact and a thicker bone layer is observed around the collagen-coated (d) than around the uncoated implants (c). Note the small osteoid layer between the bone and implant seen with higher magnification (200 $\times$ in c and d).

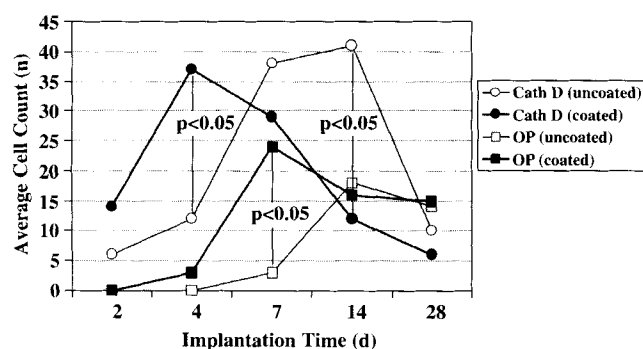


Fig. 5. Average count of cells staining against cathepsin D (circles) and osteopontin (squares) per low power field. Statistical significant differences between collagen-coated (black) and uncoated (white) implants were found as indicated with bars.

the uncoated implant and 69.5% for the collagen-coated implant. These values were obtained from 500 μ CT slices with a slice resolution of about 10 μ m.

Discussion

Titanium and its alloys, especially Ti6Al4V, are attractive materials for orthopedic applications. Titanium alloys have a high corrosion resistance, high fatigue strength, and low elastic modulus [14,18]. They display a high biocompatibility resulting in good osseointegration with high bone-implant contact [39]. Foreign body reactions are rarely observed [17] and anti-inflammatory properties are suggested [3].

Loosening of orthopedic implants, like prostheses and external fixation devices, with adverse sequelae like infection, necrosis, pain and impairment of function is a major concern in orthopedic and reconstructive surgery [42]. Besides changes in implant design, implant composition, and surgical technique, surface modification of orthopedic implants has been subject of intensive investigations. The latter include chemical modifications (oxide films, passivation, nitriding), physical modifications (heat treatment, surface-finishing), variation of

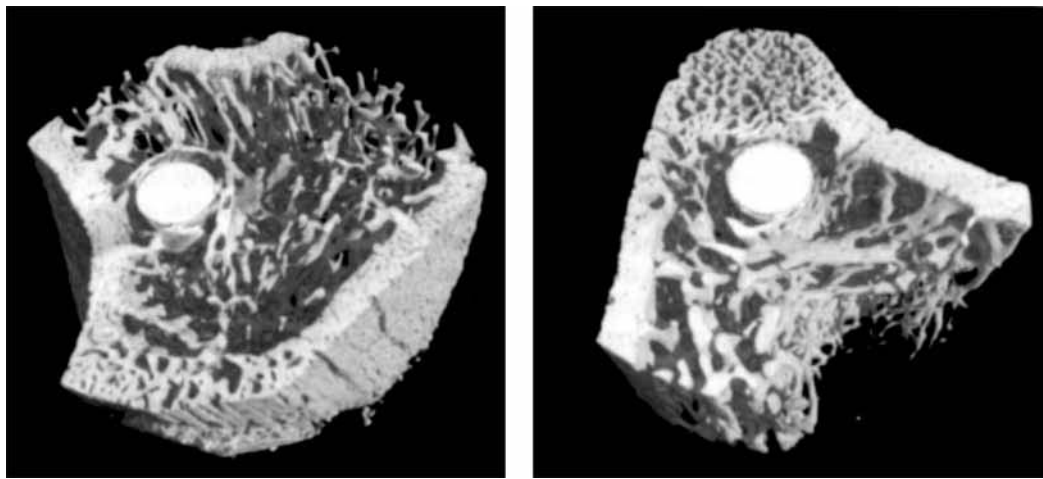


Fig. 6. Microcomputed tomographs (SR μ CT) of the metaphyseal section of the rat tibia with inserted uncoated (left) and collagen-coated (right) pins. The pins appear white, mineralized bone appears gray.

roughness (e.g. grit-blasting, porous coating, polishing), and coating of implants [18]. Numerous experimental and clinical studies have shown improved osseointegration and enhanced fixation of hydroxyapatite (HA)-coated implants. Possible applications are external fixation devices [23], pedicle screws [36], and total hip [9], knee [28] or ankle replacement [2]. There are, however, several drawbacks associated with HA coating of surgical implants. Problems include osteolysis and acetabular loosening [33], complete loss of the HA coating in retrieved specimens [34], third body wear due to HA particles from implant coating [24], and femoral bone loss with increased fracture rates [31].

The beneficial properties of type-I collagen, the major structural protein in bone, on bone regeneration are well recognized. It has been reported to be osteoconductive both as an allograft and xenograft in various biomedical applications [7,8], while the immune response to collagen does not necessarily predict adverse clinical sequelae [12]. A bovine collagen matrix enhanced proliferation of cultured osteoblasts, while a decreased cell growth was observed on several HA ceramics [47]. In a rat tibia model the addition of type-I collagen enhanced bone remodeling around a HA-based bone cement [32]. Clinical studies in dentistry have documented that type-I collagen serves as a scaffold for guided bone regeneration around HA implants [7,8]. In a rat femoral defect model, purified fibrillar collagen alone increased union rates from 0–5% to 20–30% [20]. Collagen fibrils generated *in vitro* like in the present study display a banding pattern comparable to the fibrils occurring *in vivo*, indicating that all information necessary for the self-aggregation processes is intrinsic to the monomers [46]. The main difference is the fact that, *in vivo*, numerous other proteins like procollagenases, other collagen types, and proteoglycans (decorin, biglycan, etc.) additionally influence generation both of fibrils and superfibrillar

structures, thus contributing to the high structural heterogeneity of the collagen fibrils found in living tissues [16].

So far, few applications deal with collagen coating of surgical implants. Collagen has been found to reduce capsule formation and enhance epithelialization around silicone implants [19]. Tatsumi et al. [41] observed improvement of early mending (repair/remodeling) and early calcification of osteoblast-like cells on HA/ β -TCP/sintered bone when coated with atelcollagen extracted from calf skin corium. Coating of titanium disks with porcine type-I collagen enhanced the initial attachment and increased the activity of human gingival fibroblasts [25]. Coating with type-I collagen derived from calf skin increased adhesion of rat calvarial osteoblasts on Ti6Al4V disks [15]. Further *in vitro* studies have shown a positive effect of type-I collagen on the differentiation of osteoblasts on Titanium surfaces [10,21]. To the best of our knowledge, so far no experience from *in vivo* studies exists on collagen coating of titanium implants for potential orthopedic applications.

In the present study, the cellular reaction on the implants appeared more intense around the collagen-coated implants at the early stages of bone healing. On the second post-operative day a small number of macrophage-like cells in the interface region exhibited a weak immunoreactivity for cathepsin D. The number of cathepsin D-positive cells and the intensity of the cell reaction increased rapidly until day 7 after implantation and then decreased again. Compared to randomly located macrophage-like cells, that had presumably not been affected by the operation, these phagocytotic cells in close contact to the implant showed an equal or even more intense immunoreactivity for cathepsin D. Cathepsin D is known to play an important role in bone remodeling under both physiologic and pathologic conditions [27]. The earlier observation of mononuclear

phagocytosing cells and the higher number of cathepsin-D-positive cells at the interface around collagen-coated titanium pins suggests an earlier onset of the bone remodeling process as compared with uncoated implants. The same tendency was observed for ED 1-positive cells. ED 1 is an intracellular marker for cells of the rat monocyte/macrophage lineage that also reacts with osteoclasts [11]. It is mainly located on lysosomal membranes and is probably homologous to human CD 68 [11,13]. The absence of multinucleated giant cells or foreign body granulomata suggests that no pathological foreign body reaction took place around either implant.

The adhesion of fibroblasts is thought to be a prerequisite for tissue integration of implants [25]. We observed a greater fibroblast activity as assessed with staining against vimentin at 4 and 7 days around the collagen-coated implants. Nagai et al. [25] noted the *in vitro* formation of lamellipodia and filopodia of gingival fibroblasts on collagen-coated titanium disks. Cells resembling “slender cells” [5] of both the fibroblast and osteoblast type were detected around the collagen-coated implants, the osteoblast type appeared not until 14 days around the uncoated implants. Osteoblast-associated extracellular matrix proteins (osteopontin, osteocalcin and osteonectin) were detected earlier and in higher number at the interface around the type-I collagen-coated implants, suggesting an early deporation of these proteins. Osteopontin and osteonectin, two non-collagenous extracellular matrix proteins, are produced in osteoblasts and osteocytes [37,38]. Both proteins are involved in fracture healing [26,37]. In addition, osteopontin interacts with osteoclasts, implicating it as a potentially important participant in bone resorption [22]. The CD 44/osteopontin receptor-ligand interaction is essential for intracellular–extracellular signal transduction. In bone, these cell–matrix interactions are thought to be involved in bone remodeling [45]. In the present study, the reaction for osteopontin and osteonectin at the interface region of the type-I collagen-coated titanium implants was evident from the second post-operative day and increased until the 7th post-operative day. These findings indicate a high osteogenic capacity of the bone–implant interface and the beginning of a bone remodeling process in the interface tissue around the collagen modified implant, as early as 2 days after implantation. As a result of these early events of bone formation 14 days after implantation clusters of newly formed woven bone could be detected around the coated implants, but not around the controls.

The adsorption of proteins including extracellular matrix components onto the metal surface is the first step of implant tissue interaction. Implant coating with cell-adhesive proteins therefore appears attractive in improving cell activity around bone implants to improve osseointegration [25]. Collagen type-I is effective in promoting cellular adhesion and spreading through

RGD sequences that are recognized by integrins [35]. It further serves as a basis for the mineral scaffold [1]. The interaction of osteoblasts with type-I collagen has led to enhanced alkaline phosphatase activity, osteoblast-associated gene expression and stimulated mineralization [6]. *In vitro* studies have shown, that type-I collagen is able to accelerate the initial adhesion of osteoblasts on Titanium disks [15]. The earlier appearance of osteoblasts at the implant surface and the earlier formation of woven bone in the present study support these findings for the first time in an *in vivo* study.

After 28 days no significant differences between the coated and uncoated implants were seen with respect to cell counts. Both implant types were surrounded to a great extent by newly formed lamellar bone without intervening fibrous tissue. Only a small osteoid layer, as characterized by Ayukawa et al. [5] with light microscopy and SEM, was noted between the bone and implant (Fig. 4). Both the direct bone–implant contact and the amount of newly formed bone were greater around the type-I collagen-coated implants, without reaching statistical significance. It appears therefore, that the faster remodeling induced by the collagen coating affects primarily the earlier stages of bone healing. This conclusion is in concordance with the hypotheses drawn from *in vitro* studies that suggested that collagen coating of titanium implants would improve the attachment of peri-implant tissue at an early stage [15,25]. For potential surgical and orthopedic applications this could possibly implicate a decreased susceptibility for infections at the vulnerable early stages of bone healing, shorter immobilization periods and earlier initiation of functional rehabilitation. It has to be noted however, that the results of the small animal experiment presented here do not necessarily allow conclusions on clinical settings, although the rat tibia model is established for investigating the *in vivo* reactions on orthopedic implants. Further experiments with larger animals and possibly human studies are needed to clarify the benefit of collagen coating for potential orthopedic applications. Surface roughness is known to enhance both collagen adhesion to titanium and cell adhesion to titanium surfaces [17,29]. In the present study surface roughness was not altered in order to investigate the sole effect of collagen coating.

The histological, immunohistochemical, and histomorphometrical findings from this study were supplemented by microtomographic scanning (SR μ CT) of two specimens. A close correlation was found between histomorphometric and microtomographic calculations. Due to the small numbers of specimens subjected to SR μ CT, no statistical analysis was performed. Image artifacts usually found in the vicinity of highly absorbing metals, like titanium, have not been observed because of the use of synchrotron radiation. These graphics provide adequate information on the bone–implant

interface and also suggest development of 3D image analysis modules to quantify the spatial bone amount around the implants.

Embedding of histological specimens in Technovit 9100N allows both morphological and immunohistochemical evaluation of the bone–implant interface on undecalcified sections. Detection of cell and matrix antibodies was consistent throughout the specimens. Negative controls without primary antibodies displayed no reaction. Plastic embedding of bone specimens is an established histological method that provides thin ground sections of less than 10 µm with good preservation of the tissue around metallic implants [13,44]. Shrinking artifacts can be minimized if acetone is not used for fixation [13]. Since some embedding media (Technovit 7200, 8100, Epon 812, etc.) display an exothermic reaction during polymerization with potential damage to the tissue and loss of antigenicity, we have used Technovit 9100N which enables cold polymerization [44]. The division of the implants prior to the definite staining allowed both the preparation of cutting and grinding sections with the implant in situ as well as the immunohistochemical characterization of the interface after removal of the implant and depolymerization [13]. The disadvantage of the latter is the potential damage to the tissue by removing the implant from the hardened specimen block and sectioning after depolymerization, as seen in some of our specimens. The degree of tissue preservation was acceptable in the present study with respect to the fibrous interface during the early observation times. Newly formed bone lamellae at the later stages were less preserved. Therefore, the horizontal cutting and grinding sections were used for histomorphometrical analysis. We are currently elaborating direct immunohistochemical staining methods on cutting and grinding sections with the implant in situ in order to alleviate the demanding workup of the specimens and to improve the quality of the sections.

Conclusions

The earlier observation and higher number of markers of bone resorption and regeneration suggests an increased bone remodeling around type-I collagen-coated titanium implants as compared with uncoated implants. A tendency towards higher bone–implant contact was seen at the end point of the observation period of 28 days in the cutting and grinding sections. Type-I collagen might be an attractive material for implant coating in orthopedic applications because of its high biocompatibility and osteoconductivity. It may further serve as a carrier for bioactive molecules. Adsorptive immobilization of collagen is stable on metallic surfaces which is crucial for surgical applications. Embedding in Technovit 9100N allows both morphological and immuno-

histochemical evaluation of the bone implant interface. Microtomographic scanning appears to provide information equivalent to histomorphometry in a pilot study.

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