Stem cell-coated titanium implants for the partial joint resurfacing of the knee

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Abstract

The goal of the present study was to evaluate the partial surface replacement of the knee with stem cell-coated titanium implants and to provide a basis for a successful treatment of large osteochondral defects.

Mesenchymal stem cells (MSCs) were isolated from bone marrow aspirates of adult sheep. Round titanium implants with a diameter of 2 × 7.3 mm were seeded with autologous MSC and inserted into an osteochondral defect in the medial femoral condyle. As controls, defects received either an uncoated implant or were left untreated. Nine animals with 18 defects were sacrificed after 6 months. Histological evaluation was performed by intravital polychrome fluorescent labelling, intravital perfusion with Indian ink, microradiographs and differential staining with toluidine blue. The quality of regenerated cartilage was assessed by in situ hybridization of collagen type II and immunohistochemistry of collagen types I and II.

In 50% of the cases, defects treated with MSC-coated implants showed a complete regeneration of the subchondral bone layer. In these cases collagen type II and only traces of collagen type I were detected. A high level of collagen type II mRNA expression compared to articular cartilage indicates regenerating hyaline-like cartilage. A total of 50% of MSC-coated and uncoated implants failed to osseointegrate and formation of fibrocartilage was observed. Untreated defects as well as defects treated with uncoated implants demonstrated incomplete healing of subchondral bone and formation of fibrous cartilage. A modified histological score according to Wakitani significantly demonstrated better results for cell-coated implants (8.8 ± 6.4) than for uncoated implants (5.5 ± 3.9) and for untreated defects (2.8 ± 2.5).

Our results demonstrate that, in a significant number of cases, a partial joint resurfacing of the knee with stem cell-coated titanium implants occur. A slow bone and cartilage regeneration and an incomplete healing in half of the MSC-coated implants are limitations of the presented method. To improve our approach and optimize the experimental parameters, further investigations are needed prior to clinical application.

Keywords: Tissue engineering; Osteochondral defects; Stem cells; Titanium implants

1. Introduction

In continuously strained joints such as the knee, articular cartilage is particularly exposed to traumatic or degenerative influences [1]. Chondral defects without involvement of the subchondral bone are currently treated by
debridement [2], abrasion arthroplasty [3], drilling according to Pridie et al. [4], microfracturing [5], and autologous chondrocyte transplantation (ACT). However, the necessary prerequisite for a successful treatment is the integrity of the subchondral bone, which gives the joint shape and provides differentiation and development of cartilage tissue due to biomechanical properties [1]. Therefore, therapy of deep osteochondral defects with a destroyed subchondral layer by one of the above-mentioned techniques [2–5] leads to formation of biomechanically insufficient fibrous cartilage. The consequence of this process is the development of degenerative joint disease, which has been demonstrated in long-term studies [6]. ACT did improve the histological results of cartilage formation in cases where defects involved the subchondral bone [7]. At present, only two types of therapy for osteochondral defects lead to the reconstruction of hyaline cartilage namely, the transplantation of osteochondral autografts (OATS) [8,9] or transfer of the posterior femoral condyle [10]. The disadvantages of these methods are the donor site morbidity and the limitations imposed by the size of the defects. Twenty percent of patients receiving OATS reported having problems at the donor site [11]. Yamashita et al. [12] could not avoid the development of osteoarthritis in five of 10 cases of osteochondrosis dissecans treated with OATS. Experimental investigations on the knee joint of sheep confirm these observations [13]. Tissue-engineered composites which had successfully been applied in isolated cartilage defects did not succeed in healing deep osteochondral defects [14,15]. Therefore, the goal of the present study was the joint resurfacing of the knee with stem cell-seeded titanium implants in order to reconstruct the subchondral bone and also the articular cartilage. Implants should provide optimal biomechanical conditions for the cells introduced to induce chondrogenic differentiation and regeneration of hyaline cartilage under the physiological milieu of the joint.

2. Materials and methods

2.1. Implants

Round cp titanium implants with a diameter of 7.30 mm and 28 drill holes (each with a diameter of 600 μm) were used (Fig. 1). The implants had been sandblasted with SiO₂ for 10 min (particle size 150–180 μm) and cleaned as described previously [16]. The surfaces of six implants were subjected to surface analysis. Surface roughness was measured by profilometry using a TKU300 profilometer (Hommelwerke, Schwennin- gen, Germany) with a diamond tip. The surface roughness measurements were taken at the surface of the implants at a length of 1.6 mm starting at the edge of each implant towards the centre. The surface topography was characterized by determining the parameters $R_u$, $R_s$ and $R_{max}$.

2.2. Cell culture

Mesenchymal stem cells (MSCs) were obtained using a standardized modified protocol according to Pittenger et al. [17]. With an 18-gauge syringe, bone marrow was aspirated from the iliac crest of six female sheep under anesthesia. Cells isolated by density gradient centrifugation were plated in DMEM (Gibco, Germany) and cultured with 10% autologous serum, 2 mmol/l L-glutamine and 50 μg/ml penicillin–streptomycin. The culture medium (complete medium) was changed twice a week. Cells were cultured at 37°C in a humified incubator with 5% CO₂. They formed symmetric colonies within 10–14 days and reached confluence after 3–4 weeks. After the second passage, the cells were inoculated in 24-well plates (100,000 cells/well). When cells reached confluence, titanium implants were placed in the wells and cultured as described above. Within 3–4 weeks, the cells migrated from the bottom of the culture dish in the channels and formed a dense network of cells and extracellular matrix [18,19]. In 600 μm channels, the cells showed the best migration behaviour, formation of a three-dimensional network [18,19] and cells provided a fast ingrowth of bone tissue [16].

Under specific culture conditions [17], the cells obtained in the described manner were able to differentiate into a chondrogenic and also into an osteoblastic lineage.

2.3. DNA content of implants

DNA content of a second implant, cultured parallel to the first implant, was photometrically measured after treatment with proteinase K. DNA isolation was performed using the DNA-Mini-Kit by QiaGen (Hilden, Germany).

2.4. Operation procedure

All animal experiments were performed with the approval of our institutional review board. Under general anesthesia, a lateral parapatellar incision was made to expose the femoropatellar joint. Three centimeters distal from the upper cartilage edge, holes of 7.25 mm in diameter and 5 mm deep were milled with DBCS® (MedArtis, Munich, Germany) in the medial trochlea of both femora (Fig. 2). Titanium implants, either coated with autologous cells or uncoated, were then implanted into the defects in the medullary channels of both femora (Fig. 2). Titanium implants, either coated with autologous cells or uncoated, were then implanted into the defects in press-fit technique or defects were left untreated as controls. In nine sheep, 18 osteochondral lesions were created as described above. For each treatment group, six defects were examined ($n = 6$).

2.5. Intravital fluorescence labelling

After implantation, the animals were injected with fluorescent agents (Merck, Darmstadt, Germany) every third week to label the process of bone formation. Injections were administered subcutaneously:
2.10. Immunohistochemistry of collagen types I and II

2.9. High-resolution radiography

2.8. Preparation for microscopy

2.7. Vascularization

2.6. Duration of experiments

All animals were sacrificed after 6 months in deep general anaesthesia.

2.5. Statistical design and analysis

Since it was not possible to apply all three treatments to each sheep, we used an incomplete block design to perform the experiment where the sheep represented the blocks. In order to achieve a balanced design, all three different treatment combinations (control and coated, control and uncoated, coated and uncoated) were replicated three times. This means that each treatment was applied to six sheep.

According to this incomplete balanced block design, an analysis of variance (ANOVA) was performed. Since the order control—uncoated implant—cell-coated implant of the three treatments represents an increasing intensity of treatment, it appeared to be reasonable to test for an increasing effect. Therefore, we used a test procedure which is particularly sensitive to an increasing trend. The level of significance was set to 2.5% as recommended by regulatory authorities for the use of one-sided trend tests.

Analysis was conducted with SAS 9.1 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Surface roughness of the implants

The mean surface roughness $R_a$ was $2.07 \pm 0.3\, \mu m$, mean $R_{\text{max}}$ was $12.94 \pm 2.01\, \mu m$ and mean $R_z$ was $10.73 \pm 1.46\, \mu m$ and did not reveal any significant differences between the implants.
3.1.1. DNA content of implants

A mean DNA content of 3.60 (+0.97) μg was measured prior to implantation.

3.1.2. Vascular supply

A good vascularization could be seen in all defects. Capillaries were detectable in the connective tissue of untreated defects. A capillary network grew from cancellous bone into the drill channels of the implants to the subchondral bone layer. Blood vessels could neither be detected in fibre cartilage nor in hyaline-like cartilage.

3.1.3. Anatomical features of defect healing

In untreated defects, regeneration of cancellous and subchondral bone was incomplete and started 3 months after operation (Fig. 3a–c). On average, less than 50% of the defect size was filled with bone tissue (Fig. 3a–c). Most parts were filled with connective tissue. Fibre cartilage formation was seen only at the superficial edges of the defect.

Defects treated with uncoated implants showed, in five of six cases, an incomplete reconstruction of the subchondral bone (Fig. 3d–f). Osseointegration was incomplete in three of six cases. The first bone formation was seen 9 weeks after implantation, started from the edges and moved towards the centre of the defects. Cartilage regeneration was incomplete with formation of fibre cartilage and vascularized connective tissue in five of six cases (Fig. 3d–f).

Cell-coated implants demonstrated in three of six cases a complete osseointegration (Fig. 3g–i). In these cases, a complete regeneration of subchondral, cortical bone with formation of hyaline-like cartilage was observed (Fig. 3g–i). The healing of defects started 9 weeks after implantation at the edges towards the centre of the defects. In the other three cases, the integration of the implants in the surrounding bone tissue was incomplete. No reconstruction of the subchondral, cortical bone layer was demonstrated. In these cases formation of fibre cartilage and connective tissue was detected.

3.1.4. Immunohistochemical staining of collagen types I and II

In untreated defects, a collagen type I rich connective tissue with an inhomogeneous alignment was detected (Fig. 4a–c). No collagen type II was found (Fig. 4a and b).

Lesions which had received uncoated implants revealed fibre cartilage or connective tissue at their surfaces with production of collagen types I and II (Fig. 4d–f). Only one defect demonstrated partial formation of hyaline-like cartilage.

Defects treated with cell-coated implants showed a complete healing of the defects in three of six cases with formation of hyaline-like cartilage and production of collagen type II (Fig. 4g–i). Only one defect demonstrated partial formation of hyaline-like cartilage.

3.1.5. In situ hybridization of collagen type II

In untreated lesions, no expression of collagen type II mRNA was observed (Fig. 4b). Small amounts of mRNA of collagen type II were found in defects treated with uncoated implants (Fig. 4e).

Cell-coated implants induced a strong signal for collagen type II expression in three of six cases (Fig. 4g–i). Compared to articular cartilage (Fig. 4j–l) a lower content of collagen type II was seen in all specimens (Fig. 4g).
3.1.6. Histological assessment and statistical analysis

In the modified score of Wakitani et al. [23] (Table 1), defects treated with cell-coated implants reached 8.8 points, defects treated with uncoated implants 5.5 and untreated defects 2.8 points (Fig. 5) with a common standard deviation of 4.3. A p value of 0.0228 was obtained by the ANOVA which shows a significant increase of the score.

Cell-coated implants with complete osseointegration reached on average 15 (±0.57) points (Fig. 5). Osseointe-
Authors indicate that not only the quality of MSCs according to Pridie, which are both clinically employed described by Ponticiello et al. Experiments of both MSCs embedded in a resorbable carrier matrix was treatment of isolated cartilaginous defects using human accelerated the healing process. Furthermore, a successful analogous to microfracturing, significantly improved and accompanied by punctual opening of the subchondral bone implantation of MSCs into deep cartilaginous defects, chondrocytes. Gun et al. demonstrated that the at present, are based on the differentiation of MSCs into cartilage tissue. MSCs have been shown to differentiate high number of MSCs, capable of differentiating into osteoblasts, fibroblasts, myocytes, adipocytes and chondrocytes in vitro, if they are cultured under appropriate environmental conditions. It is commonly known that healing of cartilage defects largely benefits from stem cell contribution. The techniques of microfracturing as well as drilling into the subchondral layer according to Pridie, which are both clinically employed at present, are based on the differentiation of MSCs into chondrocytes. Gun et al. demonstrated that the implantation of MSCs into deep cartilaginous defects, accompanied by punctual opening of the subchondral bone analogous to microfracturing, significantly improved and accelerated the healing process. Furthermore, a successful treatment of isolated cartilaginous defects using human MSCs embedded in a resorbable carrier matrix was described by Ponticiello et al. Experiments of both authors indicate that not only the quality of MSCs but also the amount is crucial for successful healing. This fact explains the significant difference in the healing behaviour between cell-coated and uncoated implants in the present study. However, there are only few reports on the healing of deep osteochondral defects by means of tissue engineering. Whenever a successful treatment of such cases by cell therapy was reported, the defects were small and superficial, which largely excluded transfer of the results to clinical situations. Although the traumatic destruction of subchondral bone in the case of osteochondral defects runs parallel to the immigration of MSCs, there is only formation of mechanically inferior fibrous cartilage. Introducing stem cell-seeded polylactid scaffolds in such defects does not provide regeneration of articular cartilage. The subchondral bone plays an important role in the differentiation process of stem cells into chondrocytes. Cartilage quality as determined by matrix composition and proteoglycan content of the tissue, depends on the biomechanical influences and forces being exerted on it. In the case of violation of the subchondral layer, the biomechanical environment is altered, finally resulting in the development of fibrous cartilage.

This study demonstrates that regeneration of hyaline-like cartilage in osteochondral defects can occur at the surface of cell-coated and osseointegrated implants. Thereby, the titanium implant provides the biomechanical support for the hydrostatic compression in the cartilage, which is of decisive importance for the differentiation of hyaline-like cartilage. As material for joint resurfacing, titanium was used because it is biocompatible, not toxic, it has a high corrosion resistance, its properties for joint replacement are well characterized and the joint surface can easily be shaped according to MRI or CT-scans. New titanium alloys are almost isoelastic to the cortical bone and are, therefore, an ideal biomechanical basis for the reconstruction of the subchondral bone layer. The disadvantage of titanium is that it is not absorbable. However, especially absorbable biomaterials such as polymers and collagens do not fulfill the biomechanical requirements for the joint resurfacing of the knee. Ceramics such as calcium phosphate, tricalcium phosphate or hydroxyapatite are stiff and often unable to retain their shape under cyclic loading, easily leading to the occurrence of microcracks.

Despite the fact that the results with an increasing intensity of treatment (control—uncoated implant—cell-coated implant) were significant, almost 50% of the implants, either stem cell-seeded or not, were not or not completely osseointegrated. Only osseointegrated implants were able to provide a basis for the regeneration of subchondral bone and cartilage. It is unlikely that the inhomogeneous nature of the results of cell-coated implants is caused by autologous cells themselves. No correlation was found between DNA content of each implant before implantation and the histological result. Although the chondrogenic and osteoblastic differentiation

\[ \text{DNA content} \]
The incomplete osseointegration of some of the implants, which is a major reason for the variability of the results, has to be noted as a limitation of the animal model presented. Despite the press-fit implantation and the associated primary firm fixation, a partial loosening of cell-coated and uncoated implants was observed. The sheep were not protected from bearing full weight after surgery, which may have led to an aseptic loosening of some of the implants. Therefore, a higher primary stability of the implants has to be demanded for future experiments. From a comparison of only osseointegrated implants with non-osseointegrated ones, it becomes evident that stem cell-coated implants show a better regeneration of bone and cartilage than implants without cell coating.

In tests carried out prior to the experiments reported herein, the implants were inserted flush with the subchondral bone level. This led to the generation of connective tissue (unpublished data). Only when the titanium implants were positioned just beneath the subchondral bone layer did osseous tissue develop on the implant with an accompanying regeneration of hyaline-like cartilage upon its surface.

The defects generated by the titanium implants of 7.3 mm in diameter are doubtless large with respect to the size of the sheep trochlea (width 23–26 mm). Regeneration of the subchondral layer only starts 9 weeks after intervention, as demonstrated by sequential polychrome fluorescent labelling. As known from other animal models, implanted stem cells do survive within the defect and actively contribute to healing processes [34]. Considering the relatively slow progress of healing, whether larger defects could be treated with the technique presented, remains to be analyzed.

Our results demonstrate the development of a partial joint resurfacing of the knee with stem cell-coated titanium implants. Further improvement of the technology appears to be feasible and must be achieved prior to any clinical application.

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