Case Report

Repair of a segmental long bone defect in human by implantation of a novel multiple disc graft

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ABSTRACT

Large segmental defects of the weight bearing long bones are very difficult to reconstruct. Current treatment options are afflicted with several limitations and disadvantages. We describe a novel approach to regenerate a segmental long bone defect in a patient using a multiple disc graft. Decellularized bovine trabecular bone discs were seeded with autologous bone marrow cells and cultured in a perfusion chamber for three weeks. Multiple cell-seeded discs were implanted to close a 72 mm defect of the distal tibia in a 58-year-old woman, and fixed by an intramedullary nail. Bone formation was assessed non-invasively by plain radiographs and 18F-labeled sodium fluoride-based co-registration of positron emission- and computed tomography (PET/CT). Bone was actively formed around the grafted defect as early as six weeks after surgery. Because the tibia was sufficiently stabilized, the patient was able to freely walk with full weight bearing 6 weeks after surgery. The uneventful two-year follow-up and the satisfaction of the patient demonstrated the success of the procedure. Therefore the use of multiple cell-seeded disc grafts can be considered as a treatment alternative for patients with segmental long bone defects.

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Introduction

A large segmental defect of a weight bearing long bone can be the consequence of an infection, a congenital deformity, a tumor, but most frequently it is the result of an open fracture [1]. Allografts and autografts such as free fibula- or iliac crest grafts are mainly used for the filling of large bone defects. Due to the limited availability of transplantable bone and frequently occurring donor site morbidity [2], current research activities are aimed at using autologous bone marrow stromal cells (BMSCs) in combination with a carrier to regenerate segmental bone defects.

BMSCs can differentiate into several specialized cell types, including adipocytes, chondrocytes, myoblasts, and osteoblasts [3]. In vivo, BMSCs demonstrate a strong bone-forming capacity [4–6]. To date, very limited knowledge exists about their use in humans [7,8]. The first repair of large bone defects in humans was reported in 2001 [8] with a follow-up study published six years later [7]. The authors described three patients who had a repair of a defect of the upper extremity and one patient who was treated at the proximal tibia.

In contrast to the little knowledge about the use of BMSCs for the repair of skeletal lesions in humans, BMSCs have been studied extensively in various small and large animal models [9–12]. In several studies, rodents were used to demonstrate the repair of small osseous defects by implanting BMSC-seeded grafts [9,10,12–14]. However, these grafts were several hundred times smaller than those required to close a long bone defect in humans. Because this difference in dimension represents a real challenge, large animal models using dogs and sheep were established [11,15–17]. Despite many differences among all studies, it was commonly demonstrated that BMSCs are critical to facilitate bridging of a bone defect [11,16,18–20].

In this article we describe the establishment, implantation, and long-term clinical evaluation of a novel cell-based multiple disc bone graft for the repair of a large segmental long bone defect in a patient.
Case summary

The patient, a 58-year-old woman, fell on icy ground and sustained an open fracture of the right distal tibia (AO-classification: 43C2.3). Three weeks later, the temporary external fixation was changed to an intramedullary stabilization, which was more convenient for the patient. Due to an infection with *Staphylococcus aureus* and *Enterococcus faecalis*, a partial resection of the infected bone was required five months after trauma. The resulting defect measured 72 mm and was maintained by a gentamycin-containing cement spacer (Fig. 1). Besides providing mechanical support and exerting an anti-infectious effect, the rationale of implanting a gentamycin-containing cement spacer was to induce the formation of a foreign body membrane (Fig. 1D), which has been reported to secrete growth factors that stimulate angiogenesis and bone formation [21]. Multiple wound debridements were performed during the following two months until the infection was completely eradicated.

In preparing the definitive cure of this large segmental defect, we considered the following strategies: Callus distraction by transporting a bone segment using an Ilizarov apparatus, transplantation of a large vascularised autograft, or implantation of a prosthesis combined with the fusion of the ankle and subtalar joints. However, the patient chose the possibility of transplanting xenogenic trabecular bone after ex vivo colonisation with autologous bone marrow stroma cells (BMSCs). Because this approach was considered experimental, the local ethics committee granted permission for a case-specific experimental treatment. A detailed written informed consent was obtained after discussion of all conceivable complications of this non life-saving procedure.

We removed the cement spacer two months after its implantation. To reach the spacer we cut the membrane (Fig. 1D) longitudinally at the medial side. Next, we reamed the medullary canal to a width of 10 mm and inserted a tibia nail (Expert Tibia Nail, Synthes®, Umkirch, Switzerland) in a non-dynamic fashion. A new cement spacer was modeled around the nail in the defect zone for a continuous support of the growth factor-producing membrane [21]. During the same procedure a *latissimus dorsi* flap was transplanted to cover the soft tissue defect. Physical therapy and partial weight bearing started immediately after surgery to improve the muscle strength and the range of motion.

**Graft preparation and implantation**

Bone marrow (80 ml) was aspirated from the iliac crest and evenly distributed onto 24 decellularized bovine trabecular bone discs (Tutogen Medical GmbH, Neunkirchen am Brand, Germany). With this method, approximately 3–4 × 10^5 cells were seeded per disc [22]. Each disc measured 20 mm in width and 4 mm in height with a central hole of 9.5 mm in diameter (Fig. 2A). We stimulated the discs with a constant flow of medium (10 ml/min, DMEM/Ham's F12 1:1 (Biochrom Berlin, Germany), 100 U/ml penicillin/streptomycin (Invitrogen, Karlsruhe, Germany), 0.5 µg/ml amphotericin B (Biochrom), 5 µg/ml ascorbic acid (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 10% autologous serum) in six sterile culture systems for three weeks (Figs. 2B and C). The autologous serum was obtained from the patient 12 and 6 weeks prior to bone marrow aspiration, stored at our local blood bank and used to avoid contact with antigens from other species.

To characterize the cells during the time of cultivation, samples were obtained from the supernatant on day 3, day 10, and day 17. Total protein and alkaline phosphatase (ALP) activity were quantified as previously described [22] to determine cell proliferation and osteoblast differentiation, respectively. Prior to implantation of the graft, we histologically analyzed several extra trabecular bone discs for a successful colonisation with BMSCs. Histological samples were processed as described previously [22] and stained with hematoxylin and eosin.

Implantation of the customized bone graft was performed seven months after the cement spacer was introduced. To completely fill out the segmental defect of 72 mm, 18 discs were placed into the gap after removal of both the nail and the cement spacer. We reintroduced the
nail, guided it through the holes of the discs (Fig. 2I), locked it distally, pushed it backwards to compress the discs, and fixed it proximally in a dynamic fashion.

Postoperative care and graft monitoring

Physical therapy and partial weight bearing was continued during the uneventful postoperative course. To assess the graft remodeling histologically, biopsy samples were obtained from the callus and the graft six months after surgery. In order to non-invasively determine the osseointegration of the graft, a $^{18}$F sodium fluoride-based co-registration of positron emission- (PET) and computed tomography (CT) was performed at the same time [23,24]. Static images were acquired 60 min after intravenous administration of 282 MBq $^{18}$F NaF. Low-dose CT acquisition was performed with 130 kV and 30 mAs. Nine bed positions were registered from the lumbar spine to the forefoot with an acquisition time of 5 min each. Transaxial tomograms were reconstructed in a 128 $\times$ 128 matrix and the CT data were subject for attenuation correction. Hounsfield Units (HUs) and the Standardized Uptake Values (SUVs) were quantified for several regions of interest (ROIs) including cortical bone (contralateral tibia, $n=56$), trabecular bone (vertebral body, $n=24$), callus adjacent to the graft ($n=32$), and the graft ($n=32$). Mean values for HUs and SUVs with their respective standard deviations were calculated. Statistical significance ($p<0.05$) was calculated for HUs and SUVs in the callus, trabecular bone, or cortical bone compared to the graft using the student t-test for unpaired samples.

Fig. 2. A decellularized bovine spongiosa disc is shown in Panel A. Four cell-seeded discs were incubated in one perfusion-culture chamber as demonstrated in Panel B. Six identical perfusion-culture systems (one system is shown in Panel C) were used to culture 24 discs in total. Each perfusion-culture system was kept in a tissue culture incubator and consisted of a sterile filter (a) for gas exchange, and a reservoir for culture medium (b). A bypass (c) prevented high peaks of pressure in the culture chamber (d). A roller pump (not shown) was connected to the system (e) to generate a continuous flow of medium (10 ml/min). A time-course of the protein concentration in the supernatant in shown in Panel D, and the alkaline phosphatase (ALP) activity is demonstrated in Panel E. Colonialisation of the spongiosa discs with BMSCs is presented by H&E-stained sections shown in Panel F (60×), Panel G (100×), and Panel H (400×). We guided a tibia nail through the holes of the discs as demonstrated in Panel I.
We regularly followed the patient in our outpatient department. Two years after implantation of the graft, the clinical success of the therapeutic approach and the satisfaction of the patient with the overall result was finally determined.

Results

The implanted spacer (Fig. 1) provided mechanical support to the defect zone and induced the formation of a pseudo-synovial membrane (Fig. 1D), which was previously shown to stimulate vessel- and bone formation [21]. Analysis of the cells during in vitro culture revealed a progressive increase in cell proliferation (Fig. 2D), while osteogenic differentiation did not occur (Fig. 2E). At the end of the in vitro culture, the spongiosa discs were successfully seeded with cells (Figs. 2F to H) and therefore suitable for grafting.

One day after grafting, the integrity and the correct positioning of the graft and the metal implants was confirmed. A profound callus formation was evident six weeks after surgery. Three months after surgery, the callus formation reached its maximum and no further improvement was seen after 15 months (Figs. 3A to L). The callus was not equally formed around the graft. A complete embedding was seen at the lateral side but only a partial consolidation was found at the medial side.

Consistent with the radiological findings, histology revealed an active remodeling of the callus but not of the graft (Figs. 3M to Q). Although the graft was infiltrated with granulocytes (Fig. 3O), no clinical or biochemical sign for an infection was seen. PET/CT was used to non-invasively evaluate bone remodeling (Figs. 3P to T). HUs were used to quantify radiodensity and standard values of the [18F] fluoride uptake served as a surrogate of osteoblast activity. HUs and SUVs of the callus, the trabecular bone, and the cortical bone were compared with the HUs and SUVs of the graft. This analysis revealed that the graft was more radiodense than trabecular bone and the callus, but it was less radiodense than cortical bone (Table 1). The graft demonstrated a radiotracer uptake similar to trabecular bone but less compared to the uptake seen in the callus. However, the radiotracer uptake of the graft was significantly higher than the uptake seen in cortical bone, suggesting that some bone formation might have also occurred in the graft (Table 1). Although the graft was not the main subject for an active bone formation as its radiodensity changed only minimally over time, the notion that some bone formation occurred also within the graft is demonstrated by PET/CT co-registration showing an active bone formation in both the callus and the graft with less activity in the graft (Figs. 3S and T).

Physical examination two years after grafting revealed non-infected and well perfused soft tissue with intact sensation except for the latissimus dorsi flap, which was numb. The range of motion of the right knee was not compromised compared to the contralateral side. The range of motion of the right upper ankle joint was limited to 0–30° (extension/flexion) due to a posttraumatic osteoarthritis. Although the grafted zone appeared radiologically and clinically stable, we decided not to remove the nail because it did not cause any problems and it was expected to provide additional mechanical support to the repaired defect. Our patient could freely walk a distance of 3 km and is enjoying a life quality that is almost as good as it was before the accident.

Discussion

We describe a novel approach for the repair and non-invasive follow-up of a large segmental weight bearing long bone defect in humans. Our results demonstrate that a cell-based system using a multiple disc graft with internal fixation is clinically applicable to consolidate a substantial bone loss with a good functional outcome, thereby improving the life quality of the patient.

Osteosynthetic defects due to congenital anomalies or trauma are often challenging to treat. Current treatment concepts often include the use of a graft. Alternatively, osteotomy followed by callus distraction can be performed. However, this procedure is lengthy, highly inconvenient for the patient and associated with considerable morbidity [25]. Graft-based concepts use autologous, homologous, and heterologous bone or bone substitutes. Autologous bone grafts can be either vascularised or non-vascularised but their harvest can cause donor site morbidity [2]. Homologous and heterologous grafts are available from bone banks, but they may carry pathogens or can provoke an immune response [26]. These limitations emphasize the need for novel bone reconstruction concepts.

In 2001, Quarto et al. described the first repair of large bone defects in humans [8]. Six years later the same group reported a follow-up study presenting three patients that underwent a repair of a defect of the upper extremity and one patient that was treated at the proximal tibia [7]. The bone defects in these patients were repaired using culture-expanded BMSCs, which were seeded onto hydroxyapatite scaffolds. External fixation was used to stabilize the grafted zone. Similar to our case, the postoperative course was without complications and a partial callus formation was mainly found at the surfaces of the grafts. The grafts were integrated into the host bone, leading to a stable fracture repair. However, a major remodeling of the grafts did not occur within several years. Although the patient with the defect of the proximal tibia is reminiscent of our case, both cases differ in several aspects. Besides the much younger age of the patient in their study, the defect was located in the mid diaphysis and was approximately half the size of the defect found in our patient. There was no infection prior to surgery, which was electively scheduled for limb lengthening and not for fracture repair. An Ilizarov apparatus was used to stabilize the implant consisting of a single hydroxyapatite scaffold seeded with culture-expanded BMSCs. Only radiographs were taken for post-operative control, which revealed fissures and cracks of the scaffold.

Compelling evidence exists that extensive ex vivo expansion of BMSCs leads to a progressive decrease in proliferation and a dramatic reduction of the in vivo bone-forming capacity, being even worse

Fig. 3. One day after surgery, plain radiographs (Panel A: anterior–posterior view and Panel B: lateral view) demonstrate the correct positioning of the graft and the metal implants. The boxed region in Panel A is enlarged in Panel C. Follow-up radiographs six weeks (Panels D–F), three months (Panels G–I), and 15 months (Panels J–L) after graft implantation demonstrate a callus formation around the graft. Anterior–posterior views (Panels D+G+J) and lateral views (Panel E+H+K) are shown. The boxed regions (Panels D+G+J) are magnified (Panels F+I+L) and demonstrate a callus formation as early as six weeks (Panel F) that further progresses until three months (Panel I), leading to a continuous callus formation around the graft. No further improvement in callus formation was seen between three and 15 months after implantation (Panel L). At all times, callus formation is more pronounced at the lateral side of the graft. Biopsies were taken from the callus (Panels M+N) and the graft (Panel O) and analyzed by H&E staining. Abundant osteoblasts (arrow in Panel M) underwent terminal differentiation and became entrapped as osteocytes in the newly synthesized matrix (asterisk in Panels M+N). Active remodeling was suggested by the presence of multi-nucleated giant cells (arrow in Panel N) that appeared to locally resorb the mineralized matrix (asterisk in Panels M+N). Neither bone-forming osteoblasts nor matrix-resorbing osteoclasts were remodeling the trabecular spongiosa (T in Panel O) in the graft, suggesting that the graft was not subject for active remodeling. Despite the presence of granulocytes in the graft (arrow in Panel O), no clinical or biochemical evidence was found indicating an acute infection. The coronal view of the PET/CT scan in Panel P demonstrates a specific 18F-NaF uptake in the right distal tibia compared to the healthy contralateral side. The region of radiotracer uptake is enlarged in Panel Q. The dotted yellow line indicates the location of the axial views shown in Panel R. The axial CT scan shown in Panel R reveals the position of the intramedullary nail (orange arrow in Panel R), the multiple disc graft (red arrow in Panel R), and the callus formation at the lateral side of the grafted zone (green arrow in Panel R). Some callus formation is also visible at the medial side. PET/CT co-registration is shown in Panel S and Panel T (magnification of the white box shown in Panel S). Both Panels demonstrate the position of the intramedullary nail (orange arrow), the graft (red arrow), osteoblast activity at the lateral side of the graft (green arrow), and most importantly a localized bone formation within the graft (blue arrow).
when BMSCs are derived from aged donors [27]. In a previous study, we determined that the main stimulus for BMSC proliferation in our system is continuous perfusion while mechanical stimulation facilitates osteogenic differentiation [28]. Based on these data, we used continuous perfusion for three weeks to stimulate BMSC proliferation, thus implanting a graft seeded with undifferentiated BMSCs that can differentiate into osteoblasts in vivo. The novelty and the advantage of our approach is the use of trabecular bone discs that are clinically approved, quality controlled, commercially available, easy to store, osteoconductive, and do not induce an immune response [29]. In addition, compared to more established single carriers, the presented multiple disc graft is easy to adjust to any defect size. A profound callus formation was seen six weeks after implantation. At three months, the graft zone was sufficiently consolidated to allow the patient to be fully mobilized. Although bone deposition was mainly seen around the graft, with emphasis of the lateral side of the implant, some remodeling was also documented within the graft. This finding is highly reminiscent of what has been reported in a sheep model [18]. One explanation for this observation could be a heterogeneous distribution of oxygen and nutrients at the site of repair, emphasizing the need for a vasculature-rich blastema in which precursor cells can be enriched to facilitate bone formation. However, given that BMSCs may encounter ischemic cues that can jeopardize their viability and given that human osteoblasts have an average half-life of only eight to ten days [18,30], it has been proposed that the main function of administered BMSCs is not to directly conduct bone formation. BMSCs rather promote a regenerative microenvironment in which host progenitors can be recruited to the site of repair and differentiate into matrix-producing osteoblasts. Thus, novel concepts leading to a new era of cell-based therapies propose the injection of BMSCs to establish a microenvironment that supports bone regeneration by recruiting host precursors [31]. In this case, we approached the fracture zone from medial and opened the membrane that had been formed around the spacer. Since this membrane is known to stimulate angiogenesis and bone formation [21] the incision might have compromised a neo-vascularization, thereby leading to a reduced callus formation at the medial side compared to the lateral side. However, since this hypothesis remains to be proven it is also possible that the distribution of mechanical strain or other factors contributed to the pronounced callus formation at the lateral side. New bone formation was assessed non-invasively using 18F-labeled sodium fluoride (18F-NaF)-based PET/CT. 18F-NaF ions are positron-emitting moieties, whose skeletal uptake occurs as fluorapatite based on the exchange with hydroxyl ions in the hydroxyapatite crystals. A strong and rapid uptake in bone, accompanied with a very rapid clearance from blood, causes a high bone-to-background ratio and a high spatial resolution [23]. This allows for a bone graft incorporation analysis [24]. The PET/CT signal was normalized to the administered dose and the body weight using standard uptake values (SUVs). SUVs were used as a measure of osteoblast activity. Hounsfield Units (HU) were determined and used as a measure of tissue density. These measurements are consistent with our clinical observation that the graft mainly served as a guide for newly formed bone.

A recently published retrospective histological analysis of biopsies taken from patients that underwent hip arthroplasty or opening-wedge tibia osteotomy followed by grafting using the same carrier we described here revealed that remnants of the graft still persisted 11 months after surgery in almost half of the patients without signs of inflammation [29]. In addition, this study also demonstrated the osteoconductive properties of this material resulting in the formation of new bone in more than half of the patients. Collectively, these data demonstrate a progressive osseointegration of the graft combined with an incomplete resorption, leading to the conclusion that the turnover of the material is less accelerated in humans compared to animals [29]. Our observations of a strong callus formation along the graft, its partial remodeling, and the absence of immune reactions are therefore consistent with these findings.

In this study we only treated one patient and the overall usefulness as well as advantages, the long-term effect of the nail, and limitations of the presented approach have to be determined in an animal model and in a long-term follow-up of a larger group of patients. Thus, we intend to treat more patients in the future with the presented approach in addition to further analyzing the role of BMSCs in graft vascularization using in vivo models. However, cell-loaded multiple disc grafts have the advantage of causing negligible donor site morbidity and might be useful in patients like ours in which conventional approaches are likely to be unsuccessful. For example, allografts have been reported to fail in the majority of the cases with very large segmental defects [32]. A callus distraction would have taken more time than our approach and it would have been inconvenient for the patient with a considerable risk of infection [25]. Vascularised fibula grafts were demonstrated to have a low osteogenic potential, presumably due to the small amount of trabecular bone [33]. The long-term recovery of our patient was uneventful, reporting no infection, failure of the graft, or loosening of the implants. Our patient was psychologically stable and able to fully cope with the treatment. She cooperated very well with the intense physical therapy program and demonstrated a strong positive attitude towards her clinical progress. She returned to normal life very quickly, participated in sports activities, and enjoyed a good life quality.

**Conclusion**

To our best knowledge, we report for the first time the repair of a large segmental defect of a weight bearing long bone in a patient using a cell-loaded multiple disc graft and internal fixation. In addition, we describe a PET/CT-based technique for the non-invasive monitoring of the osseointegration of the graft. Given that our patient was in a poor clinical condition at the beginning of the treatment, we consider our approach as encouraging. We are confident that further development of current regenerative approaches will open new possibilities in modern orthopaedic surgery.

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