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Influence of the induced membrane filled with syngeneic bone and regenerative cells on bone healing in a critical size defect model of the rat's femur

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ABSTRACT

Introduction: The induced membrane technique for the treatment of large bone defects consists of a 2-stage procedure. In the first stage, a polymethylmethacrylate (PMMA) cement spacer is inserted into the bony defect of a rat's femur and over a period of 2–4 weeks a membrane forms that encapsulates the defect/spacer. In a second operation the membrane is opened, the PMMA spacer is removed and the resulting cavity is filled with autologous bone.
Since little effort has been made to replace the need for autologous bone this study was performed to elucidate the influence of different stem cells and the membrane itself on bone healing in a critical size femur defect model in rats.
Especially the question should be addressed whether the use of stem cells seeded on a β-TCP scaffold is equivalent to syngeneic bone as defect filling in combination with the induced membrane technique.
Materials and Methods: A total of 96 male Sprague-Dawley (SD) rats received a 10 mm critical size defect of the femur, which was stabilized by a plate osteosynthesis and filled with PMMA cement. In a second step the spacer was extracted and the defects were filled with syngeneic bone, β-TCP with MSC + EPC or BM-MNC. In order to elucidate the influence of the induced membrane on bone defect healing the induced membrane was removed in half of the operated femurs. The defect area was analysed 8 weeks later for bone formation (osteocalcin staining), bone mineral density (BMD) and bone strength (3-point bending test).
Results: New bone formation, bone mineral density and bone stiffness increased significantly, if the membrane was kept. The transplantation of biologically active material (syngeneic bone, stem cells on b-TCP) into the bone defect mostly led to a further increase of bone healing. Syngeneic bone had the greatest impact on bone healing however defects treated with stem cells were oftentimes comparable.
Conclusion: For the first time we demonstrated the effect of the induced membrane itself and different stem cells on critical size defect healing. This could be a promising approach to reduce the need for autologous bone transplantation with its' limited availability and donor site morbidity.

Introduction

Whenever we are confronted with large bone defects, different techniques are available to treat those. Small defects up to 5 cm can be healed by autologous bone grafts. If this limit is exceeded, more extensive methods are needed like the vascularized bone transfer, bone transport with the Ilizarov technique [1] or vascularized periosteal flaps [2–6]. Furthermore, several biodegradable bone graft substitutes have been developed and are commercially available [7–9].

Masquelet et al. developed and described the induced membrane concept for reconstruction of long bone defects

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[10,11]. This is a two-staged technique by which defects up to 25 cm can be restored. In a first step a PMMA (polymethyl methacrylate) cement spacer is brought into the bone defect and a membrane is built around it. This induced membrane is vascularized and contains various growth factors, depending on its maturation state. Furthermore, it is assumed that the induced membrane supports the re-vascularization of the later bone graft and prevents resorption [12–14]. In a second step the induced membrane is incised and the cement spacer is removed.

In clinical practice, the membrane tube is then filled with cancellous bone and in the following months a complete bone healing can be frequently observed with adequate function [11,12].

Histological and biomechanical characteristics of the induced membrane have already been investigated. It is proven by Pelissier et al. that growth factors like VEGF, TGF-beta and BMP-2 are secreted and that the membrane presents high vascularization in rabbits [12]. This group also proved that proliferation and differentiation of osteoblasts is stimulated.

These results were confirmed by our group in previous publications. We established a critical size femoral defect model in Sprague-Dawley (SD) rats to investigate the induced membrane after plate osteosynthesis for longer periods of time [13]. We could already proof that the induced membrane undergoes changes during time. Stem cells found in the membrane after 2 weeks were reduced after 4 and 6 weeks and the amount of senescent cells increased. This was followed by structural changes like an increase in density and structuring of collagenous fibres. Vascularization significantly improved in the same time [13].

The successful use of autologous bone graft for defect filling in combination with the induced membrane was reported by Klaue et al in a 3 cm stable diaphyseal femoral defect in sheep [14]. However, to circumvent the limitations of autologous bone graft, tissue engineered bone defect fillings on the basis of scaffolds combined with regenerative cells should also be considered. In our own previous work we were able to demonstrate a significant improvement of bone defect healing by transplantation of myeloid endothelial progenitor cells (EPC) and marrow stromal cells (MSC) in a femur defect model of the male athymic rat. Vascularization improved significantly when EPC were seeded to the scaffold (β-tricalcium phosphate, β-TCP) and brought into the defect zone but without associated callus formation. In contrast, MSC seeded scaffold resulted in elevated callus formation, nevertheless the mechanical stability did not increase. In contrast the combination of MSC and EPC led to a significant improvement of stability, which could be proven radiologically and biomechanically [15–19]. The disadvantage of this approach is its' incompatibility with the clinical demands in an emergency surgery department due to the long cultivation period of both cell types.

Hence, utilization of mononuclear cells taken from isolated iliac crest bone marrow aspirate is a new approach [20–22]. In contrast to EPC and MSC, bone marrow mononuclear cells (BM-MNC) can be extracted, isolated and re-transplanted within 24 h in a clinical setting. It has been shown recently that the transplantation of BM-MNC seeded on a β-TCP scaffold into a critically sized femoral bone defect of the rat led to a bone defect healing similar to the bone healing mediated by EPC and MSC presumably by improvement of early vascularization as we demonstrated previously in another animal study [6,23].

We hypothesized, that the combination of regenerative cells with the induced membrane technique could lead to a further improvement of cell based bone-healing approaches, since the induced membrane serves as a" bio-reactor" to supply the defect zone with the implanted stem cells. Additionally, immigrating cells and growth factors from the induced membrane might reinforce bone healing and enhance vascularization.

Thus, the aim of this study was to investigate the effect of the induced membrane in combination with syngeneic EPC and MSC or syngeneic BM-MNC on the bone healing response in comparison to syngeneic bone using our well-established critical size bone defect model of the rat.

Material and methods

Animal care

All experiments were performed in accordance with regulations established and approved (Project No. F3/23; Regierungspräsidium, Darmstadt, Germany) by our institution's animal care and oversight committee according to German law. Twelve-week-old male SD rats (Janvier, Saint Berthevin, France) weighing 350–400 g were used. Animals were kept in individual cages in temperature (21 °C), light (12 h light 12 h dark), and air flow controlled rooms and fed standard rodent chow and water ad libitum. Rats were monitored daily in the postoperative period for any complications or abnormal behavior.

EPC and MSC harvest, isolation and characterization

Early EPCs were isolated from the spleen of syngeneic donor rats (male SD rats, n = 5) as described previously [24]. Briefly, the donor rats were killed by intraperitoneal injection of pentobarbital [500 mg/kg body weight]. The spleen was recovered and transported in PBS supplemented with 2% penicillin/streptomycin (Biochrom, Berlin, Germany) to the laboratory. The spleen was cut into small pieces (approximately 3 mm) and gently mashed using syringe plungers. The cell suspension was filtered through a 100 μm mesh (BD Biosciences, Heidelberg, Germany), washed once with PBS and layered onto a ficoll density gradient (1.077 g/mL; Biochrom) and centrifuged (30 min, 900 g). Recovered mononuclear cells were washed twice with cold phosphate buffered saline (PBS $^{w/o}$) (10 min, 900 g), and each 2×10^6 cells per cm^2 were cultivated on individual fibronectin-coated (10 μg/mL; Sigma, Deisenhofen, Germany) wells of 6-well culture dish in 1 ml of endothelial basal medium (Cambrex, Verviers, Belgium) supplemented with endothelial growth medium (Cambrex) single quots at 37 °C, 5% CO2. After 48 h, nonadherent and weakly adherent cells were removed, the medium was changed, and the cells were cultivated for additional 72 h. A parallel preparation was performed to evaluate the percentage of endothelial-differentiated cells. EPCs were identified using the method previously described. Briefly, cells were incubated for 1 h with 2.4 μg/ml DiLDL (1.1′-dioctadecyl-3.3.3′.3′-tetramethylindo-carbocyanine-labeled acetylated low-density lipoprotein, Cell-Systems, St. Katharinen, Germany) in EBM supplemented with 20% FCS. Thereafter, cells were fixed with 2% paraformaldehyde for 10 min, and after washing with PBS $^{+/+}$, FITC labeled Ulex europaeus agglutinin-1 (10 μg/ ml) (lectin, Sigma®) was incubated for 1 h. Cells presenting fluorescence, lectin) were considered to be EPCs. For the experiments the cells were detached by accutase treatment (10 min, PAA Laboratories, Linz, Austria), washed once PBS, and subsequently adjusted to a density of 2.5×10^5 cells in 100 μl and subjected to the cell seeding procedure to the β-TCP scaffold as described later.

MSCs were obtained from femurs of three donor rats (SD rats) as described previously [25]. Femurs were removed and cleaned. The condyles were cut using a site cutter and the bone marrow was flushed with a sterile syringe filled with PBS supplemented with 1% penicillin and streptomycin (P/S, Biochrom). The cells were recovered and washed once with PBS. The cell pellet was re-suspended in DMEM supplemented with 10% FCS and 1% P/S and directly transferred to a 75 cm^2 culture flask. One culture flask per

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