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Mesenchymal stromal cell implantation for stimulation of long bone healing aggravates *Staphylococcus aureus* induced osteomyelitis

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ABSTRACT

Large bone defects requiring long-term osteosynthetic stabilization or repeated surgeries show a considerable rate of infection. Mesenchymal stromal cells (MSCs) have been successfully used to enhance bone regeneration, but their powerful immunomodulatory effects may impose an enhanced risk for osteomyelitis development. In order to unravel whether implantation of MSCs aggravates a simultaneous bone infection, a hydrogel-supported osteomyelitis ostectomy model was developed in which rats received a femoral bone defect with rigid plate-fixation. After fibrin-assisted transfer of *Staphylococcus aureus* (SA), effects of MSC implantation on osteomyelitis development were quantified over 3–4 weeks.

All SA-infected animals developed an acute local osteomyelitis with significantly increased blood neutrophil count, abscess formation and bone destruction. MSC-treatment of infected defects aggravated osteomyelitis according to a significantly elevated osteomyelitis score and enhanced distal bone loss with spongy alteration of cortical bone architecture. Increased attraction of macrophages, osteoclasts and regulation of pro- and anti-inflammatory mediators were potential MSC actions.

Overall trophic actions of MSCs implanted into non-sterile bone defects may enhance an infection and/or exacerbate osteomyelitis. Studies on antibiotic carrier augmentation or antibiotic treatment are warranted to decide whether MSC implantation is a safe and promising therapy for orthopedic implant-stabilized bone defects at high risk for development of infection.

1. Introduction

The closure of large bone defects after trauma or tumor resection remains a challenge in orthopedic surgery. Although the incidence of infection following elective bone surgery is low, patients with open fractures or large lesions are known to be at high risk for development of infection [1], especially when long-term osteosynthetic stabilization and repeated surgeries are indicated. Osteomyelitis is defined as a microbial colonization of the bone associated with inflammation and bone destruction [2]. It is a feared complication with devastating consequences especially since orthopedic implants stabilizing the defect cause an even higher susceptibility for bone infection due to bacterial colonization and biofilm formation [3]. Osteomyelitis often requires long-term antibiotic therapy, may entail implant removal and multiple revisions and has a 1 in 50 chance to lead to amputation [4]. It is therefore of great interest to improve strategies for treatment of large bone defects.

With an incidence of up to 50%, *Staphylococcus aureus* (SA) is the major pathogen found in bone infections causing severe and chronic forms of osteomyelitis. SA is capable of bone cell invasion, can express virulence factors and form metabolically inactive small colony variants (SCV) with a capacity for long-term persistence [5]. Next to bacterial factors, the susceptibility for SA-induced bone infection depends on additional risk factors by the host including disorders of the immune system [6].

Studies on bone marrow-derived mesenchymal stromal cell (MSC) implantation as a supportive measure in bone defect treatment have provided promising results [7,8]. MSCs can easily be isolated from the bone marrow [9], have a high proliferation capacity and express the surface markers CD90, CD105 in the absence of CD34 and CD45 [10,11]. The ability of clonal MSC populations for

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in vitro differentiation into chondrogenic, adipogenic and osteogenic lineages was used as a proof for their multipotent differentiation capacity [12]. MSCs have successfully been used for the treatment of bone defects in humans [13,14] and animals [15,16] with or without a supportive biomaterial [17].

Next to their function as progenitor cells with a capacity to contribute to newly forming bone by osteogenic differentiation [9], a trophic, pro-angiogenic and immunomodulatory action of MSCs was established [18–20], which activates endogenous regenerative mechanisms [21]. We recently provided evidence that MSCs stimulate early long bone healing via enhanced attraction of macrophages and endothelial cells and promote angiogenesis via enhanced VEGF expression. While no evidence was obtained for immunosuppression by MSCs over the first 6 days, it remains an open question whether MSCs may exert an immunosuppressive or immunomodulatory effect on bone healing in the long run [22].

Creating an immunosuppressive local microenvironment in a bone defect by implantation of MSCs seems undesirable since this may confer an increased risk for the establishment of bone infections in non-sterile defects. Thus, it is mandatory to evaluate whether MSCs exert a beneficial effect on bone regeneration also in an environment in which a very small SA contamination may lead to osteomyelitis or whether MSC implantation will even carry a risk to enhance the genesis and progression of a bone infection due to trophic and/or immunomodulatory actions.

The aim of this study was to assess whether MSC implantation influences the establishment and progression of infection in SA-contaminated bone defects with regard to inflammatory status, bone destruction and cellular reaction. We are the first using a hydrogel-supported osteomyelitis ostectomy model enabling a precise implantation of a defined number of SA into a plate-stabilized femoral bone defect. Our model allowed us to study whether a very small contamination of an orthopedic implant-stabilized bone defect with a classical osteomyelitis agent is a contra-indication for MSC implantation. We further searched for mechanisms of crosstalk between MSCs and SA and the local environment.

2. Materials and methods

2.1. Animals and study design

A pilot study with six female Sprague–Dawley rats (14 or 22 weeks of age, 289 ± 18 g; Charles River, Sulzfeld, Germany) was first conducted to find the lowest dosage of SA (10^4, 10^3 or 10^2 CFU per bone ending) to induce local osteomyelitis in the used model. In the main study male Sprague–Dawley rats (n = 28, 14–16 weeks of age, 526 ± 48 g; Charles River, Sulzfeld, Germany) were included. The experimental protocol was approved by the local animal experimental ethics committee and all procedures were performed according to the national guidelines for animal care and in accordance with the European Union Directive (2010/63/EU). Animals were housed in groups of two in Makrolon Type 4 cages in a conventional animal facility with 12-h light/dark cycle, 22 °C room temperature, 55% air humidity and ad libitum access to water and food (Ssniff, Soest, Germany). Health status of the animals was monitored as recommended by the Federation of Laboratory Animal Science Associations (FELASA) using sentinel animals. Three rats were used for rat MSC donation. Twenty-five animals received a 2 mm mid-diaphysal full-thickness bone defect in the right femur. Three experimental groups were defined with an uninfected control group receiving only fibrin carrier (n = 5 animals, 3 weeks) and two infected groups receiving SA one of which was additionally treated with MSCs implanted in fibrin hydrogel (n = 10 animals for each group with n = 5 animals at 3 and 4 weeks, respectively). Animals were sacrificed 3 or 4 weeks after surgery under general anesthesia by final blood withdrawal and right femora were dissected. Blood counts were determined for each animal and femora were scanned by micro-computed tomography (μCT) and then subjected to histology. One animal of the control group had to be excluded from data analysis due to a spontaneous infection by another germ. In vitro co-cultivation experiments of human MSCs with SA were performed to investigate the influence of SA on MSC viability and gene expression.

2.2. Isolation and cultivation of MSCs

Rat MSCs were isolated from the bone marrow of 3 rats using a modified centrifugation method [23] and characterized as described [22]. In short, rat MSCs were extracted from tibiae and femora by centrifugation at 750×g for 2 min. Cells were pooled and cultivated in 0.1% gelatin-coated flasks in expansion medium consisting of high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 12.5% fetal calf serum (FCS), 2 mM L-glutamine, 1% non-essential amino acids, 0.1% 2-mercaptoethanol (Invitrogen, Karlsruhe, Germany), 100 U/ml Penicillin, 100 μg/ml Streptomycin (Biochrom, Berlin, Germany) and 4 ng/ml recombinant human fibroblast growth factor-2 (Active Bioscience, Hamburg, Germany). Surface marker expression and differentiation capacity were determined. After passage 1, cells were frozen in liquid nitrogen and re-cultivated for 4 days before implantation.

For in vitro investigations, human bone marrow samples were extracted from three orthopedic patients receiving a total hip replacement. Written consent was obtained from all subjects and the study received approval from the local ethics committee. Human MSCs (hMSCs) were isolated by Ficoll-Paque™ Plus density gradient centrifugation (GE Healthcare, Munich, Germany) and seeded at a density of 125.000 cells per cm^2 in the same expansion medium used for rat MSC cultivation. After 24 h, non-adherent cells were removed. Cells were cultivated under standard conditions (37 °C, 6% CO_2) up to passage 3 and characterized according to MSC criteria [10]. Cells were then frozen in liquid nitrogen and re-cultivated 3 days before in vitro experiments.

2.3. SA cultivation

The SA strain UAMS-1 previously isolated from a patient with chronic osteomyelitis was obtained from the American Type Culture Collection, Manassas, VA (ATCC number 49230). UAMS-1 is known to establish chronic bone infections in rodent models [24–26]. SA stocks were kept at −80 °C on ceramic beads (Mikrobank™, pro-lab diagnostics, ON, Canada). Two days before use one bead was plated on blood agar (BD, Heidelberg, Germany) and cultivated over night at 37 °C. Next day one colony was re-plated on blood agar over night at 37 °C. At the day of surgery, colonies were transferred into 8 ml tryptic soy broth (TSB; BD, Heidelberg, Germany) at 37 °C to obtain bacteria in log-phase growth. After 2.5 h, 100 CFU per ml were adjusted by spectrophotometry (Densimat, bioMerieux, Marcy l'Etoile, France).

2.4. Preparation of SA and MSCs for implantation

A fibrin hydrogel (Baxter, Vienna, Austria) was selected as carrier for controlled delivery of a defined number of SA and MSCs. The fibrinogen component was diluted 1:2 and the thrombin component 1:50 in PBS. Animals were treated with two cavity clots transferred into both open bone endings containing SA, SA + MSCs or no additives according to Table 1. Per cavity clot, 100 CFU SA were delivered within 15 μl of the fibrinogen component and kept on ice until implantation to stop bacterial growth. MSCs were prepared separately for every animal to standardize time between harvesting and implantation to around 20 min.

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