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The effect of BMP-7 gene activated muscle tissue implants on the repair of large segmental bone defects

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ABSTRACT

Background: This study was conducted in order to investigate the effect of Bone Morphogenetic Protein-7 (BMP-7) transduced muscle cells on bone formation and to further develop an innovative abbreviated *ex vivo* gene therapy for bone repair. As conventional *ex vivo* gene therapy methods require an elaborative and time-consuming extraction and expansion of cells we evaluated an expedited approach. Fragments of muscle tissue were directly activated by BMP-7 cDNA and implanted into bone defects.
Methods: 25 male, syngeneic Fischer 344 rats were used in the present study. Muscle tissue was harvested from two donor rats and either transduced with an adenovirus carrying the BMP-7 cDNA or remained unmodified. 5 mm osseous defects in the right femora of 23 rats were treated with either unmodified muscle tissue (control group) or BMP-7 activated muscle tissue (treatment group). Six weeks after surgery, rat femora were evaluated by radiographs, micro-computed tomography (μCT) and histology.
Results: Implantation of BMP-7 activated muscle grafts led to bony bridging in 5 out of 12 defects (41.7%) and to bone formation without bridging in 2 out of 12 defects. In 2 femoral defects of this group radiographs, μCT-imaging and histology did not reveal significant mineralization. Three animals of the BMP-7 treatment group had to be euthanized due to serious wound infection. The bone volume of the treatment group was significantly ($p = 0.007$) higher compared to the control group.
Conclusion: This study shows that BMP-7 gene activated muscle fragments have the potential to regenerate critical-size segmental bone defects in rats. However, further development of this promising expedited treatment modality is required to improve the healing rate and to investigate if the high infection rate is related to treatment with BMP-7 activated muscle grafts.

Introduction

Currently, the standard treatment modality for the repair of bone defects is autologous iliac crest bone grafting. This approach does not represent an ideal solution due to substantial donor site morbidity [1–3]. Other methods for the treatment of bone defects include allograft transplantation [4], and distraction osteogenesis [5]. However, it has been reported that such surgical approaches for bone defect repair lead to an unsatisfactory outcome in a significant number of cases [5–7]. For these reasons there is an increasing interest in osteoinductive stimulation of bone repair.

Marshall Urist's report in the 1960s about the osteoinductive capacity of demineralized bone matrix was the beginning of an intensive search and study of osteoinductive bone growth factors [8]. Today we know that growth factors, such as recombinant, human bone morphogenetic protein-7 (BMP-7), have a strong

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effect on migration [9], differentiation [10], and proliferation [11] of precursor cells. The FDA approved BMP-7 in 2001 for treatment of long bone non-unions and a few years later for lumbar spinal fusion as an alternative to bone autograft [12]. Although clinical studies demonstrated effectiveness of BMP-7 [13–15], we need to optimize the way of delivering recombinant growth factors due to the difficulty of retaining the molecules at the application site and their short biological half-life [16,17].

Gene therapy offers a sophisticated way of growth factor delivery and can overcome problems associated with protein therapy. Transduction of cells with cDNAs encoding growth factors can lead to a production of these therapeutic agents for an extended period of time at the application site [18–20]. Especially the non-integrating adenoviral vector appears to be a suitable gene delivery vehicle for tissue repair as it allows efficient transduction of a variety of cells and transgene expression is only transient lasting several weeks [21,22].

In vivo BMP-7 adenoviral gene transfer has been applied in recent bone repair studies primarily by implanting vector-carrying scaffolds [13–15,23–25]. This approach resulted in enhanced bone healing in animal models and data from these studies are promising. However, drawbacks associated with *in vivo* gene delivery are the difficulty of targeting specific cells and the risk of inducing an immune-response to the vector. Therefore, it is unlikely that *in vivo* gene therapy will be used in a clinical setting for treatment of tissue lesions in humans unless these safety issues have been addressed.

Ex vivo gene therapy approaches are not associated with above named safety problems as gene transfer occurs outside of the body. Specific cells can be selected, transduced under controlled conditions and inserted within a tissue lesion. BMP-7 producing fibroblasts [26], bone marrow derived cells [27,28] and fat derived cells [29] have induced bone growth in preclinical studies. So far, for *in vivo* bone repair studies, muscle cells have been genetically engineered to produce BMP-2 [30] and BMP-4 [31] but not BMP-7.

The aim of this study was to evaluate the effect of BMP-7 producing muscle cells on the repair of critical-sized segmental bone defects in a well-established rat model. To do this, however, we did not apply a conventional *ex vivo* gene therapy method that requires the isolation and expansion of autologous cells. Instead, we used a less cumbersome, expedited *ex vivo* gene therapy strategy, which has been successfully employed in previous studies for the delivery of BMP-2 to bone defects [32,33]. To further develop this novel approach and to evaluate its potential for bone repair BMP-7 cDNA was directly transferred to muscle tissue fragments using an adenoviral vector. The gene activated muscle grafts were then implanted into large segmental bone defects in rats and their potential to regenerate bone was investigated using established evaluation methods [32,33].

Materials and methods

Study design

We used 25 male syngeneic Fischer 344 rats (weight 220–250 g) in this experiment. Muscle tissue was taken from two euthanized donor rats. In the right hind limb of 23 rats a critical sized mid-femoral defect (5 mm) was created and stabilized by an internal fixator (KTK Kunstofftechnik, Germering, Germany). Animals were divided in two groups. Osseous lesions were treated with either muscle grafts activated by an adenoviral vector carrying the human BMP-7 cDNA (Ad.BMP-7) ($n = 12$) or untreated muscle grafts ($n = 11$). Six weeks postoperatively repair of the femora was evaluated by radiographs, micro-computed tomography (μCT) and histology.

Generation of adenoviral vector particles

The adenoviral BMP-7 vector was constructed by the group of Renny Franceschi by Cre/*lox* recombination, as described earlier [26,34]. Briefly, full-length BMP-7 cDNA was cloned into the *Bam*H1 and *Kpn*1 sites of a pAd*lox* shuttle plasmid which was then cotransfected with psi5 adenovirus into CRE8 cells. After 5 days, the primary lysates were collected in order to reinfect the CRE8 cells. The virus was then purified from this secondary lysate by the plaque assay method. The presence of the BMP-7 insert in each lysate was verified by using polymerase chain reaction. Amplification of positive plaques was performed in 293 cells and subsequently purified chromatographically using ViraBind™ Adenovirus Purification Kit (Cell Biolabs Inc.). Infectious titres were determined *via* immunohistochemical detection of the adenoviral hexon protein in infected HEK 293 cells.

Tissue harvest

The animals used in this study (Fischer 344 rats) are genetically identical (syngeneic) and are widely used for transplantation studies. Using this well established syngeneic rodent autotransplant model means that transplantation of harvested muscle tissue from one animal to another does not elicit a host-*versus*-graft immune response [35–37]. For practical reasons we mimicked autograft transplantation in this experimental setting.

From the upper thighs of two sacrificed donor animals muscle tissue was cut out. This harvested tissue was then washed with phosphate buffered saline and put into a Petri dish. Subsequently, the tissue was cut into slices of approximately 1 mm thickness. Then tissue discs were punched out using a 4 mm dermal biopsy punch creating muscle tissue fragments of a standardized size. No cell isolation was performed. Discs were placed in 24-well plates.

Activation of tissue grafts

Muscle tissue grafts were infected with 1×10^8 plaque forming units (pfu) of Ad.BMP-7 or remained untreated according to their group affiliation. After diluting the adenoviral vector particles appropriately in Dulbeccos's modified Eagle's medium (DMEM) (BiochromeAG, Berlin, Germany), a micro pipette was used to drip 10 μL of the vector solution on the surface of the muscle tissue fragments. The tissue pieces were then incubated together with the viral vector for 1 h at 37 °C. After that 700 μl DMEM was added to each well and the muscle tissue was placed back in the incubator for 1–4 days prior to surgery and implantation into bone defects.

Measurement of BMP-7 production by ELISA

A portion of the created muscle discs was used for an *in vitro* measurement of BMP-7 up-regulation by ELISA (Quantikine, BMP-7 Immunoassay, R&D Systems, Wiesbaden, Germany). Four muscle discs per well were cultured in 700 μl of DMEM (in a 24-well plate). Media was changed every third day. Supernatants of BMP-7-transduced muscle tissue were harvested on days 3, 9 and 30 and BMP-7 concentration was determined. These measurements were performed in triplicate.

Surgical procedure

The operative and experimental procedures were approved by the institutional animal ethics committee. We used a modified version of a previously established critical-sized, femoral defect rat model [38–40]. Rats were anesthetized by intraperitoneal administration of xylazine (10 μg/g of body weight) and ketamine (90 μg/g of body weight). After aseptical preparation for surgery,