THE EFFECT OF RECOMBINANT HUMAN VASCULAR ENDOTHELIAL GROWTH FACTOR ON PREVENTING ATROPHIC NONUNION FORMATION

INTRODUCTION

We have demonstrated that periosteal cauterization at the fracture site produced atrophic nonunion and immediate local administration of recombinant human bone morphogenetic protein-7 (rhBMP-7) could completely prevent nonunion formation in rats. Vascular endothelial growth factor (VEGF) is important for endochondral ossification and essential for normal angiogenesis and appropriate callus formation in fracture healing. Given the importance of endogenous VEGF in normal fracture healing, treatment with exogenous VEGF may be expected to improve angiogenesis and bone regeneration in fracture healing. Prior studies have shown that local administration of VEGF enhanced bone formation in a mouse challenged fracture which was created by stripping periosteum at the fracture site and in a rabbit critical-sized bone defect. VEGF and BMP-4 have also been shown to work synergistically in bone regeneration and repair. Other reports, however, have shown that VEGF failed to influence blood flow and quality of the bone regenerate in rabbit distraction osteogenesis and VEGF alone without a combination with BMP-4 did not work well in mouse bone regeneration and repair. The potential of exogenous VEGF on bone regeneration and repair is still controversial.

The purpose of this study is to evaluate whether recombinant human VEGF (rhVEGF) administration to the fracture site has a potential to prevent the atrophic nonunion formation.

METHODS

Animal model: Three-month-old male Long Evans rats were used in this study under a research protocol approved by the Institutional Animal Care and Use Committee. The experimental atrophic nonunion model was created according to the previously reported method. Briefly, a femoral mid-shaft fracture was created with three-point bending. The fracture site was exposed and the periosteum was cauterized circumferentially for a distance of 2mm on each side of the fracture.

Immediate administration of rhVEGF: rhVEGF (rhVEGF165) suspended in rat tail tendon collagen buffer (total volume: 25µl) was administered to the fracture site immediately after the periosteal cauterization. The doses of 10, 50, 100, and 250µg were tested (n=3 per treatment, total 12 animals).

Continuous administration of rhVEGF: A continuous administration of rhVEGF was performed using a subcutaneous implanted osmotic pump (Alzet®, DURECT). We used a polyethylene catheter to deliver rhVEGF to the fracture site locally. Pumps which have delivering durations of 7 days and 14 days were used, and total doses of 100µg and 1,000µg were tested. Thus, we have four groups which are 7 days-100µg, 7days-1,000µg, 14 days-100µg, and 14days-1,000µg (n=3 per each treatment, total 12 animals). The pumps were removed immediately after the delivery periods.

Evaluations of fracture healing: Fracture healing was evaluated by radiographs taken at two, four and six weeks. Animals were sacrificed at six weeks and harvested femurs were subjected to histological evaluation and measuring torsional stiffness.

Assay of rhVEGF activity: The activity of the rhVEGF were confirmed by the MTS assay (Promega) using human umbilical vein endothelial cells before use. The activity of rhVEGF remaining in the pump after the delivery was also assayed.

Gene expression profile of VEGF in the nonunion model: Gene expression profile of VEGF in the nonunion model was compared with that in the standard healing fracture (closed femoral mid-shaft fracture). Four animals from each group were sacrificed at post-fracture days 3, 7, 10, 14, 21, and 28 (total 48 animals). The newly generated tissues, i.e., fracture callus for the standard fracture and fibrous tissue surrounding the fracture site for the nonunion were harvested. Total cellular RNA was extracted from the harvested tissue and reverse-transcribed to synthesize cDNA samples. TaqMan® primer and probe pairs for VEGF and GAPDH were from Applied Biosystems. Real-time quantitative PCR was done in triplicate on the cDNA with an ABI 7700 Sequence Detector. Results were normalized to GAPDH levels using the formula ΔCt (threshold cycle) = Ct of VEGF – Ct of GAPDH. The comparative Ct method was used to investigate the amount of target gene relative to a calibrator. Of forty-eight animals, an arbitrary animal was selected as a calibrator. The ΔCt value was calculated as follows; ΔCt is the Ct of an animal – ΔCt of the calibrator. The amount of VEGF normalized to GAPDH and relative to a calibrator of an animal was given by the formula 2^(-ΔΔCt).

Statistical analysis: The values of gene expressions of standard fracture and nonunion were compared at each time point and evaluated with Student’s t-test. Significance was defined as p-values less than 0.05.

RESULTS

Immediate and Continuous administration of rhVEGF to the nonunion model: Radiographs at six weeks showed no evidence of bridging callus with either administration methods. Nonunion was confirmed histologically and biomechanically at six weeks in all animals.

Gene expression profile of VEGF in the nonunion model: The gene expressions of VEGF in the nonunion were significantly lower (*) than in the standard fracture at post-fracture days 7, 10, 14, and 21.

DISCUSSION

The timing of administration and dose are expected to influence on VEGF activity. In the first part of our study, immediate administration of rhVEGF could not prevent the nonunion formation. The activity of rhVEGF was confirmed and various doses were tested including a relatively high dose. Therefore, we judged that a single immediate administration of rhVEGF has no obvious effect and wondered whether more prolonged administration was needed.

As the second step, we tried a continuous administration of rhVEGF, but again found that nonunion formation could not be prevented. As a positive control, we administered rhBMP-7 using the same pump. It successfully achieved bony union, therefore, our method of continuous administration was confirmed to be reliable. Moreover, we harvested the implanted pumps and the remaining rhVEGF in the pumps maintained its activity. Literature suggests that excessive VEGF may lead to detrimental effects on bone regeneration, and one explanation for our negative results may be that our dosage was too high.

In addition to these results, it is known that the expression of VEGF is highest on post-fracture day 5 or 10 in fracture callus and is abundant in fracture hematoma in normal fracture healing. In order to better understand the time points at which VEGF may be important in bone healing, we investigated the gene expression profile of VEGF in the nonunion model and compared it with that in the standard healing fracture. The gene expression profiles suggest that the low VEGF gene expressions after the post-fracture day 7 may have a role in the nonunion formation.

The supplementation of VEGF in the initial phase of fracture healing may be unnecessary and after the post-fracture day 7 may be the optimal time point for administration. This will be explored in subsequent studies.

REFERENCES


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