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Expression of VEGF Gene Isoforms in a Rat Segmental Bone Defect Model Treated with EPCs

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Abstract

Angiogenesis and osteogenesis are essential for bone growth, fracture repair, and bone remodeling. Vascular endothelial growth factor (VEGF) has an important role in bone repair by promoting angiogenesis and osteogenesis. In our previous study, endothelial progenitor cells (EPCs) promoted bone healing in a rat segmental bone defect as confirmed by radiological, histological, biomechanical and microCT evaluations. While EPCs have demonstrated effectiveness in animal models of fracture healing, the mechanism by which EPCs enhance fracture healing remains unclear. We hypothesized a possible paracrine mechanism of action, where the secretion of growth factors critical to the processes of fracture healing (such as VEGF), is responsible for the positive effects of EPC therapy. The purpose of this study was to evaluate VEGF gene expression after local EPC therapy for a rat segmental bone defect.

Methods: Rat bone marrow-derived EPCs were isolated by the Ficoll-paque gradient centrifuge technique. The EPCs were cultured for 7 to 10 days in endothelial cell growth medium with supplements and collected for treatment of the rat segmental bone defect. EPCs were identified by immunocytochemistry staining with primary antibodies for CD34, CD133, FLK-1, and vWF. A total of fifty six rats were studied. A five millimeter segmental bone defect was created in the middle 1/3 of each femur followed by mini plate fixation. The treatment group received 1×10^6 EPCs locally at the bone defect on a gelfoam scaffold and control animals received the gelfoam scaffold only. Seven control and seven EPC treated rats were included in each group at 1, 2, 3 and 10 weeks. Animals were sacrificed at the end of the treatment period, and specimens from the fracture gap

area were collected and immediately frozen. Rat VEGF mRNA was measured by reverse transcriptase-polymerase chain reaction (RT-PCR) and quantified by VisionWorksLS. All measurements were performed in triplicate.

Results: Cultured EPCs at 1 week showed positive staining for CD34, CD133, Flk-1 and vWf markers. The EPC group had a greater VEGF expression than the control group at weeks 1, 2 and 3, but not at week 10. Three VEGF isoforms were detected in this rat model: VEGF₁₂₀, VEGF₁₆₄ and VEGF₁₈₈. VEGF₁₂₀ and VEGF₁₆₄ levels peaked at two weeks, while VEGF₁₈₈ levels peaked at three weeks. All three VEGF isoform levels were low at ten weeks.

Discussion and Conclusion: EPC-based therapy for a segmental bone defect results in increased VEGF expression during the early period of fracture repair. In addition, the specific VEGF isoform may be a key regulator of the bone healing process. These findings demonstrate that EPCs may promote fracture healing by increasing VEGF levels and thus stimulating angiogenesis, a process that is essential for early callus formation and bone regeneration.

Key Words: Vascular Endothelial Growth Factor (VEGF), Endothelial Progenitor Cells (EPC), Fracture Healing, Angiogenesis

Introduction

Fracture of bone disrupts its circulation and leads to acute necrosis and hypoxia of adjacent bone and marrow. Severe fractures can have a compromised ability to heal that corresponds to the degree of soft tissue injury, periosteal stripping, and bony comminution. Angiogenesis and osteogenesis are essential for bone growth, fracture repair, and bone remodeling. Endothelial Progenitor Cells (EPCs) and Vascular Endothelial Growth Factor (VEGF) have important roles in bone repair by promoting angiogenesis and osteogenesis. In our previous studies, we have shown that EPCs promoted bone healing in a rat segmental bone defect as confirmed by radiological, histological, and microCT evaluations.¹ In addition, the EPC treatment of fractures resulted in a significantly higher strength by biomechanical examination.² In separate investigations, we demonstrated that cell-based VEGF gene transfer is effective in the treatment of segmental bone defects in a rabbit model and an in vitro cell culture model.^{3,4} Previous study has suggested that VEGF plays its most important role during the early phase of fracture healing and that VEGF concentrations decrease after five days.⁵

While EPCs have demonstrated effectiveness in animal models of fracture healing, the mechanism by which EPCs enhance fracture healing remains unclear. We hypothesized that EPCs enhance fracture healing by paracrine mechanisms, secreting growth factors critical to the processes fracture healing, such as VEGF. The purpose of this study was to better understand how EPCs enhance osteogenesis and angiogenesis by determining if

there is an increased expression of VEGF after local EPC therapy for a rat segmental bone defect.

Methods

Rat bone marrow-derived EPCs were isolated by the Ficoll-paque gradient centrifuge technique, as previously described.¹ The EPCs were cultured for 7 to 10 days in endothelial cell growth medium with supplements (EGM-2-MV-SingleQuots, Clonetics), and collected for treatment of the rat segmental bone defect. EPCs were identified by immunocytochemistry staining with primary antibodies for CD34, CD133, Fetal Liver Kinase-1 (Flk-1), and Von Willebrand factor (vWF).

A total of fifty six rats were studied. A five millimeter segmental bone defect was created in the middle 1/3 of each femur followed by mini plate fixation. The treatment group received 1×10^6 EPCs on a gelfoam scaffold locally at the bone defect and control animals received saline-soaked gelfoam only. Seven control and seven EPC treated rats were included in each group at 1, 2, 3 and 10 weeks, for a total of 28 animals in each group. Animals were sacrificed at the end of the treatment period, and specimens from the fracture gap area were collected and immediately frozen with liquid nitrogen.

The total mRNA was extracted (Trizol™, Invitrogen) and rat VEGF mRNA were measured by reverse-transcriptase polymerase chain reaction (RT-PCR) using the RNeasy RNA isolation kit (Qiagen) and quantified by VisionWorksLS. Primer sequences to detect rat VEGF isoforms were sense 5'-CTGCTCTCTTGGGTGCACTGG-3' and antisense, 5'-CACCGCCTTGGCTTGTCACAT-3'. This pair of primers generated a

different sized product for each of the splicing forms of VEGF mRNA. The predicted PCR products for the major forms, rVEGF₁₂₀, rVEGF₁₆₄ and rVEGF₁₈₈ were 431, 563 and 635bp, respectively.⁴ Equal RNA loading and blot transfer was assessed by stripping and reprobing the same membranes with a probe against 18S ribosomal RNA. All measurements were performed in triplicate.

Statistical analysis: ANOVA was used to test for significant differences between treatment groups and control groups, and a p-value of <0.05 was considered statistically significant. Statistical analyses were performed with SPSS Base 15.0 for Windows © 2007 (SPSS Inc. Chicago, IL, USA).

Results

Characterization of EPCs using immunocytochemistry staining: the cultured EPCs at 1 week showed positive staining vs. no staining in controls (without primary AB) for CD34, CD133, Flk-1 and vWF markers (Figure 1).

Three rat VEGF isoforms were detected in this cell culture model: rVEGF₁₂₀, rVEGF₁₆₄ and rVEGF₁₈₈. We compared the endogenous rVEGF₁₂₀, rVEGF₁₆₄ and rVEGF₁₈₈ expression level of the tissue at the fracture sites that were treated with EPCs versus controls (Figure 2). The EPC treated group showed greater expression of rVEGF₁₂₀, 164 and 188 than controls at week 1, week 2 and week 3. VEGF₁₂₀ and VEGF₁₆₄ levels peaked at two weeks, while VEGF₁₈₈ levels peaked at three weeks. All three VEGF isoform levels were low at ten weeks. Cumulative rVEGF expression was higher in the EPC treated

group than the non-treated controls at weeks 1, 2 and 3 ($p<0.005$) but not at week 10 (Figure 3).

Discussion

Historically, the enhancement of fracture healing has been limited in terms of treatment options. Recent advances in understanding the biology of fracture healing and the importance of both angiogenesis and osteogenesis have opened new avenues for enhancing repair in a clinically useful manner. Most attention has focused on the potent angiogenic and osteogenic properties of certain growth factors and cells. Bone is a highly vascularized tissue that relies on the close spatial and temporal connection between blood vessels and bone cells to maintain skeletal integrity. Angiogenesis is an essential component of bone healing and skeletal development.

EPCs were first described and defined by Asahara et al.⁶ They are bone marrow-derived cells with the ability to differentiate into endothelial cells and to participate in the establishment of neovasculature mainly by indirect, paracrine mechanisms. EPCs have been shown to express various endothelial surface markers (such as CD34, VEGFR2, and CD133), and to migrate to sites of tissue ischemia.⁷ These findings constituted the basis for the use of ex vivo expanded EPCs as a novel therapeutic option to augment neovascularization in animal models of myocardial infarction and limb ischemia.⁸ Blood flow recovery and capillary density were markedly improved in a number of investigations^{9,8} following EPC treatment. Fractures may induce mobilization of EPCs from bone marrow and recruitment of these mobilized EPCs to the fracture site, thereby augmenting neovascularization and thus osteogenesis during the process of bone healing.

Lee et al¹¹ investigated EPC mobilization during bone regeneration and the possible contribution of EPCs to increased vascularization and new bone formation during distraction osteogenesis in mouse and rat tibias. The signals from the bone regeneration site mobilize EPCs from the bone marrow into the peripheral circulation. The investigation and therapeutic application of EPCs in orthopaedic trauma is a relatively novel idea. Our research group has recently reported success with the application of bone marrow derived-EPCs to segmental bone defect models in rat studies.^{1,2} In addition, blood-derived EPCs have been used to enhance bridging of sheep tibia critical sized defects.¹² These early results suggest that EPCs may be ideally suited for cell-based therapy for fracture healing and angiogenesis. Moreover, it has been shown recently that EPCs are capable of differentiating into osteogenic cells in vitro, and can be up-regulated in response to orthopaedic trauma in humans.¹³

While there is clear evidence in animal models of fracture healing of the beneficial effects of EPCs, the mechanism by which EPCs enhance fracture healing remains poorly understood. We hypothesized that EPCs may enhance fracture healing by paracrine mechanisms, secreting growth factors critical to the process of fracture healing, such as VEGF. VEGF is of particular interest because of its ability to induce angiogenesis.^{3,14} Inhibition of VEGF activity delays bone repair in mice, decreases blood flow, and leads to non-unions in rabbits.¹⁵ VEGF has another important role in bone repair by stimulating and affecting chemotaxis, proliferation, survival, and activity of major skeletal cells, including osteoblasts,^{4,14} osteoclasts,¹⁶ and chondrocytes.¹⁷ VEGF promotes segmental bone defect healing and affects microarchitectural differences of new bone formation.³

In our current study we sought to test our hypothesis of a paracrine mechanism of action for EPC therapy by comparing VEGF expression in EPC treated bone defects versus controls. First, we were able to demonstrate that we had obtained an appropriate population of EPCs from our differential culture technique by confirming positive staining for known EPC markers with our immunocytochemistry results. Second, we were able to demonstrate that EPC-based therapy for a segmental bone defect results in increased VEGF expression during the early period of fracture repair. In addition, specific VEGF isoforms may be key regulators of the bone healing process, and these results require further investigation.

These results suggest that EPC therapy increases the expression of VEGF at the fracture site, particularly in the early phases of fracture healing. This corresponds to the period in fracture healing when VEGF levels are the most critical. These results lend support to our hypothesis of a possible paracrine mechanism of action for EPC therapy in fracture healing. Fracture healing is a complicated and multicellular process that involves a cascade of numerous growth factors and it is likely overly simplistic to attribute the success of EPC therapy to increased expression of a single growth factor. However, we feel that these results support the concept of a paracrine mechanism of action for EPC therapy and encourage further investigation of other growth factors such as the bone morphogenetic proteins (BMPs).

Conclusion:

These findings demonstrate that EPCs may promote fracture healing by increasing VEGF levels and thus stimulating angiogenesis and osteogenesis, a process that is essential for early callus formation and bone regeneration.

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Figure Legends

Figure 1. Characterization of EPCs using immunocytochemistry staining: the cultured EPCs at 1 week showed positive staining vs. no staining in controls (without primary AB) for CD34, CD133, Flk-1 and vWF markers.

Figure 2. The expression of VEGF mRNA in an EPC treatment rat femur defect at 1, 2, 3 and 10 weeks. RT-PCR bands of VEGF isoforms with the expected mRNA size of 431-bp for rVEGF₁₂₀, 563-bp for rVEGF₁₆₄ and 635-bp for rVEGF₁₈₈ respectively, and relative levels of VEGF mRNA isoform expression calculated against 18S at week 1, 2, 3 and 10.

Figure 3. Cumulative rVEGF isoform expression was higher in treatment groups versus controls at 1, 2 and 3 weeks ($p < 0.05$). There was no significant difference at ten weeks between the two groups.