Vascular Endothelial Growth Factor Is More Important Than Basic Fibroblastic Growth Factor During Ischemic Wound Healing

Claudio J. Corral, MD; Aamir Siddiqui, MD; Liancun Wu, MD; Catherine L. Farrell, PhD; David Lyons, PhD; Thomas A. Mustoe, MD

Objectives: To test the influence of vascular endothelial growth factor (VEGF) on normal and ischemic wounds in a noncontractive dermal ulcer standardized model in the rabbit ear and to assay the levels of both VEGF and basic fibroblastic growth factor messenger RNA levels in normal and ischemic wounds at different intervals during the healing process.

Design and Interventions: Dermal ulcers were created in the normal and ischemic ears of 20 anesthetized young female New Zealand white rabbits. Either VEGF 121, VEGF 165 (30 µg per wound), or buffered saline solution alone was applied to each wound and covered. Wounds were harvested at day 7 or 10 and evaluated histologically. Twenty-four similar rabbits were wounded in the same manner and their untreated wounds were harvested at 1, 3, 7, and 10 days after wounding. The wounds were analyzed with reverse transcriptase polymerase chain reaction.

Main Outcome Measures: Histologic specimens were measured for amount of new epithelium and granulation tissue. Reverse transcriptase polymerase chain reaction was used to determine basic fibroblastic growth factor and VEGF messenger RNA expression.

Results: Both isoforms of VEGF improved granulation tissue formation in both normal and ischemic wounds with a magnitude similar to other vulnerary agents tested in the past. Vascular endothelial growth factor application had no effect on new epithelium formation. In contrast to basic fibroblastic growth factor, VEGF messenger RNA levels were induced 4 fold by ischemia alone and 6 fold by wounding in both ischemic and normal wounds.

Conclusion: Vascular endothelial growth factor seems to be more important than basic fibroblastic growth factor during ischemic wound healing. Treatment of ischemic wounds with VEGF improves the deficit in wound healing produced by ischemia.

Arch Surg. 1999;134:200-205

Wound healing is characterized by an ordered sequence of events in response to injuries that ultimately result in tissue formation, reepithelialization, and scar formation. Angiogenesis is an integral part of this sequence of events and occurs during the proliferative phase of wound healing. It is characterized by new vessel formation and vascular hyperpermeability that improve tissue oxygenation and nutrient delivery and promote cellular and matrix deposition in the wound.1

Several growth factors have been implicated as possible mediators of angiogenesis during wound repair, including transforming growth factor beta-1, tumor necrosis factor alpha, epidermal growth factor, keratinocyte growth factor, interleukins 1, 6, and 8, basic fibroblastic growth factor (bFGF), platelet-derived growth factor (PDGF),2,3 and most recently, vascular endothelial growth factor (VEGF).4-6 These growth factors have angiogenic properties in vitro and in vivo. Their angiogenic properties have been traditionally categorized as either direct or indirect based on endothelial cell activation and mitogenesis.7,8 Of these, only bFGF, PDGF, and VEGF have been shown to be directly mitogenic to endothelial cells7,9; however, it remains unclear which of these growth factors is the most important mediator of the angiogenic phase of healing. This question is of primary clinical importance, since improving the angiogenic response during wound healing using a single growth factor or in combination, may partially reverse the healing deficit observed in ischemic wounds.

Several authors have described possible mechanisms by which these growth factors are angiogenic.7-9 There is evi-
MATERIALS AND METHODS

ANIMAL WOUNDING AND VEGF TREATMENT

Young adult female New Zealand white rabbits (New Franken Inc, New Franken, Wis) weighing 2.7 to 3.4 kg, were acclimated and housed under standard conditions and were used in all experiments. All surgical procedures were performed under sterile conditions as previously described. All animal protocols were approved by Northwestern University Animal Care and Use Committee.

Twenty rabbits were divided into ischemic and nonischemic groups. Rabbits were anesthetized with ketamine hydrochloride (60 mg/kg) and xylazine hydrochloride (5 mg/kg) prior to surgery. In the ischemic group, the ears were made ischemic by dividing the rostral and central arteries and the entire dermal circulation of the ear. Three full-thickness, 6-mm, dermal punches were made down to bare cartilage with the aid of a binocular microscope. In the nonischemic group, 4 wounds were similarly made in each ear. The wounds were treated at the time of wounding with either recombinant VEGF 121, VEGF 165 (30 µg per wound) (AMGEN Corp, Thousand Oaks, Calif), or phosphate-buffered saline solution vehicle. All wounds were covered with an occlusive dressing (Tagaderm; 3M, Minneapolis, Minn) for the duration of the experiment.

Ischemic wounds were harvested at day 10 and nonischemic wounds at day 7 as previously described. All wounds were excised, bisected, and fixed in neutral buffered 10% formaldehyde solution (Sigma Chemical Co, St Louis, Mo). The specimens were then dehydrated in graded alcohol and xylene, embedded in paraffin, and sectioned (3 µm), taking care to obtain a cross section as near as possible to the center of the wound.

HISTOLOGICAL STAINING

All slides were stained with hematoxylin-eosin using standard protocols. The stained sections were analyzed under ×40 magnification. All slides were examined by 2 independent observers (C.J.C. and A.S.) with standard light microscopy under ×40 magnification. New granulation tissue formation and new epithelium were measured using a 100-µm lens redicle on the light microscope at 30 magnification. All slides were examined by 2 independent observers (C.J.C. and A.S.) with standard light microscopy under ×40 magnification. New granulation tissue formation and new epithelium were measured using a 100-µm lens redicle on the light microscope at 40 magnification. The redicle was sectioned into 10-µm² squares, and the area occupied by the tissue was measured.

RNA EXTRACTION AND COMPETITIVE REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

After wounding, 24 rabbits were divided into 4 groups as to number of days after wounding: day 1, day 3, day 7 and day 10. All rabbits were wounded as above; however, only 1 ear was made ischemic. All wounds were left untreated and were covered with the occlusive dressing for the duration of the experiment. At the appropriate time, the rabbits were anesthetized and a 7-mm punch biopsy specimen of skin was obtained from each ear to serve as an ischemic and a normal skin control. Granulation tissue was harvested with a 7-mm punch biopsy specimen and immediately immersed in liquid nitrogen for later guanidine thiocyanate/phenol–based reagent to isolate RNA from the sample (TRI Reagent, Cincinnati, Ohio) as previously described. Total RNA was pooled from each ear and quantified with a spectrophotometer (Genequant model 80-2103-98; Biochrom, Cambridge, England) prior to complementary DNA (cDNA) conversion. Five micrograms of total RNA was converted to cDNA using Moloney-Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) and random primers (Gibco Biological Research Laboratories, Grand Island, NY) as previously described.

Rabbit VEGF primers were designed from conserved Genbank sequences (AMGEN Corp). Amplimers obtained with these primers were sequenced to assure the correct target gene was being amplified. The FGF rabbit primers were obtained from a Genbank rabbit sequence (AMGEN Corp). Glutaraldehyde phosphate dehydrogenase rabbit primers were kindly donated by US Surgical Corporation, New Haven, Conn. Reverse transcriptase polymerase chain reaction was performed as previously described. Serial dilutions of MIMIC and constant concentration of cDNA were coamplified using a polymerase chain reaction. These products were run in a 2% agarose gel, photographed under UV light, and analyzed by densitometry (Imaging Densitometer GS-670; Bio Rad, Richmond, Calif). The ratio of target product to MIMIC was then obtained as previously described. Glutaraldehyde phosphate dehydrogenase amplification was used as an external control of the reverse transcriptase reaction, and this was run in all cDNA samples tested. Each reaction was run in duplicate starting from the RNA to the cDNA step.
The precise role VEGF plays in the wound healing process is unknown; however, given its known biological activity, VEGF could potentially be playing a prominent role in the repair process. In the following set of experiments, we tested the effect of exogenous VEGF 121 or VEGF 165 treatment in both ischemic and nonischemic wounds in the rabbit ear dermal ulcer model. These 2 isomers were chosen since they are readily available and seem to be the most biologically active in vivo. We assayed VEGF and bFGF mRNA levels in normal and ischemic skin and in wounds to highlight any possible differences between these growth factors in their response to an ischemic event.

---

**RESULTS**

**EFFECT OF EXOGENOUS VEGF ON WOUND HEALING**

Treatment of rabbit ear wounds with 0.1, 1.0, and 10 µg per wound of either isomer of VEGF had no statistically significant effect (data not shown), although there was a trend with higher doses for 10% to 15% improvement in new granulation tissue formation and epithelium formation (Figure 1). Neither VEGF isomer had an effect on new epithelium formation (Figure 2).

Figure 1. New granulation tissue formation in response to 30 µg per wound of vascular endothelial growth factor (VEGF) in nonischemic (A) and ischemic (B) wounds. Note the difference in scale between the 2 graphs.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nonischemic Wound (N=40)</th>
<th>Nonischemic VEGF 121 (n=20)</th>
<th>Nonischemic VEGF 165 (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P&lt;.01</td>
<td>10.0</td>
<td>7.5</td>
<td>5.0</td>
</tr>
<tr>
<td>P&lt;.01</td>
<td>10.0</td>
<td>7.5</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Figure 2. New epithelium tissue formation in response to 30 µg per wound of vascular endothelial growth factor (VEGF) in nonischemic (A) and ischemic (B) wounds. Note the difference in scale between the 2 graphs. No significant differences were found.

---

**EFFECT OF ISCHEMIA AND WOUNDING ON bFGF AND VEGF mRNA LEVELS**

To control for the reverse transcriptase step in our polymerase chain reaction protocol, every cDNA sample was assayed for glutaraldehyde phosphate dehydrogenase expression. We found no significant difference in glutaraldehyde phosphate dehydrogenase expression in any of the cDNA samples used (Figure 4). Ischemia up-regulated VEGF mRNA expression 3- to 5-fold over nonischemic skin. The effect was seen as early as 1 day following the onset of ischemia and lasted through the duration of the experiment with a trend toward normalization (Figure 4, B). Wounding the skin up-regulated VEGF mRNA expression 6- to 7-fold, irrespective of whether the wound was ischemic. This effect was also seen at day 1 after wounding and persisted for 10 days after wounding (Figure 5). Basic fibroblastic growth factor expression was not significantly induced by ischemia, in fact there was a 2-fold reduction of bFGF mRNA levels in ischemic skin with a trend toward normalization by day 10 after wounding. Wounding alone increased bFGF mRNA levels less than 2-fold over normal skin in both ischemic and nonischemic wounds (Figure 6).

---

**COMMENT**

During wound healing an ordered sequence of events takes place that promotes the repair of injured tissue.
sues. This cascade of events is triggered by injury and ultimately leads to tissue regeneration, matrix deposition, and scar formation. Angiogenesis is a critical component of the wound healing response, with several growth factors potentially mediating the angiogenic response.

We have previously shown that bFGF, PDGF-BB, and transforming growth factor beta-1 have angiogenic properties in healing wounds and also promote granulation tissue formation, matrix deposition, and re-epithelialization through different mechanisms.15-17 Of these, bFGF and PDGF have a direct mitogenic effect on the endothelial cell and are considered to have direct angiogenic properties.7 We have also shown with exhaustive experiments that bFGF improves wound healing in nonischemic wounds, but fails to do so in ischemic wounds.16,17 Thus, the role of bFGF in impaired wound healing remains controversial. Basic FGF seems to improve wound healing in nonischemic models of impaired healing such as diabetic mice and steroid-treated animals30,31; however, a large clinical trial of bFGF in leg ulcers was stopped because no therapeutic effect was detected.32 In 1995, Richard et al33 in 1995 concluded that topical application of bFGF had no advantage over placebo for healing chronic neuropathic diabetic ulcers of the foot. It seems that bFGF may not be effective in impaired models of healing where ischemia is the culprit of the impairment. In light of these data, VEGF may be a more important regulator of angiogenesis and may help to promote wound healing in ischemic wounds. As shown by others, we were also able to detect a 2-fold decrease in bFGF mRNA levels in response to ischemia, an observation that may provide clues as to why bFGF fails to enhance wound healing in ischemic wounds despite improved angiogenesis, and it suggests that bFGF is not the prime mediator of angiogenesis in ischemic conditions.7,34 We have also previously observed that bFGF retained biological activity within ischemic wounds when the rabbits were treated with hyperbaric oxygen.17 These findings imply that oxygen-dependent signaling mechanisms may be required for bFGF activity in hypoxic wounds, and they highlight the potential importance of VEGF as a hypoxia-inducible factor during wound healing.

Vascular endothelial growth factor has biological properties that make it a more attractive modulator during ischemic wound healing than either bFGF or PDGF. Vascular endothelial growth factor mRNA transcription is induced by a variety of growth factors and cytokines. We have shown that VEGF mRNA transcription is not only induced by ischemia in rabbit skin but is also induced in a greater extent by tissue injury. The levels of VEGF mRNA were higher in wounded tissue (6- to 8-fold), irrespective of the...
All rabbit wounds treated with doses of either VEGF 121 or VEGF 165, of 30 µg per wound showed a notable increase over controls in the amount of granulation tissue formation. As expected, the magnitude of the effect was doubled in ischemic wounds. The fact that exogenous VEGF still had an effect on rabbit ear wounds despite maximal transcriptional up-regulation implies that either endogenous VEGF levels are not enough to saturate all VEGF receptors or exogenous VEGF has a local autoinductive effect on its own receptors at the wound site. Vascular endothelial growth factor receptors have been found to be up-regulated during wound healing and have been shown to be up-regulated in the rat lung in response to ischemia. Receptor up-regulation may account for the increased effect of exogenous VEGF in ischemic wounds. Recent data from our laboratory suggest that receptor regulation may be more important than growth factor regulation during wound healing.

Increases in angiogenesis alone cannot entirely explain the observed improvement in granulation tissue, since bFGF fails to promote healing in ischemic wounds despite marked angiogenesis. Among the other known biological properties of VEGF, vascular hyperpermeability in the wounded tissue may play a more prominent role during the healing process than previously described. Vascular hyperpermeability improves the efficiency of plasma protein delivery and cellular migration into the area of injury and is not a biological property of bFGF. Moreover, the endothelial cell is known to secrete a host of biologically active growth factors and cytokines from the endothelial cell, has an important influence on the cascade of events necessary for normal and effective wound repair.

This work was supported in part by grant GM-41303, from the National Institutes of Health, Bethesda, Md (Dr Mustoe). Presentcd at the Wound Healing Society Meeting, Minneapolis, Minn, May 1995, and Boston, Mass, June 1996.

Corresponding author: Thomas A. Mustoe, MD, Division of Plastic and Reconstructive Surgery, 707 E Fairbanks Ct, Suite 811, Chicago, IL 60611.