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The effect of vascular endothelial growth factor on the healing of ischaemic skin wounds

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Vascular endothelial growth factor; Skin; Wound healing; Angiogenesis; Tensile strength

Summary The effect of exogenous vascular endothelium growth factor (VEGF) on wound healing in an ischaemic skin flap model was evaluated in this study. Seventy-two Sprague–Dawley rats were used. Normal incisional wound and H-shaped double flaps were used as the wound models. The study was divided into two parts. In Part I, VEGF protein levels were determined from the incisional and H-shaped ischaemic wounds at 12 and 24 h, postoperatively. In Part II, tensile strength and immunohistochemical stains were examined to determine the level of microvessel density (MVD) at 1 and 2 weeks, postoperatively in simple incisional wounds, ischaemic wounds, and ischaemic wounds following 1 ml (1 μg/ml) exogenous VEGF injections into the subcutaneous tissue. The results showed a significantly higher level of VEGF protein in the ischaemic wounds than the incisional wounds. Tensile strength was statistically higher in the incisional wound group and in the ischaemic flap wounds with VEGF treatment compared to the ischaemic flaps with no treatment at 1 week, postoperatively ($p > 0.05$). MVD data indicated that ischaemic wound repair with VEGF treatment had significantly higher MVD than the normal incisional wounds and ischaemic wounds without treatment. We conclude that exogenous application of VEGF can increase early angiogenesis and tensile strength in the ischaemic wound.

During the wound healing processes, an abundant blood supply is necessary to meet the enormous local demands of debridement, fibroblast proliferation, extracellular matrix synthesis, and epithelialisation.[1–3] Impairment of blood supply may be a contributing factor in delayed healing, or nonhealing, in chronic wounds such as diabetic foot ulcers, pressure ulcers, and wounds caused by chronic and acute arterial occlusion.[4,5]

Recent advances in the understanding of neovascularisation have made angiogenesis a prime target for therapeutic manipulation in wound healing. Efforts have been made to induce or stimulate new blood vessel formation to reduce the unfavourable tissue effects caused by local ischaemia or to enhance tissue repair.[6–8] Growth

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factors, which are now known to play roles in cell division, migration, differentiation, and enzyme production, are also important regulators of wound angiogenesis.[9,10] Consequently, intense interest is now focused on the pharmacological application of angiogenic growth factors in the compromised wound.[8,11-15]

Angiogenesis is a complex and multistage process, which is controlled by a variety of factors. Vascular endothelium growth factor (VEGF) is a potent direct angiogenic factor that stimulates in vitro endothelial cell migration and activation, and in vivo angiogenesis.[16-18] In this study, we evaluated the effect of VEGF on an ischaemic wound-healing model in rats. The strength and angiogenesis of healed wounds were examined after administration of exogenous VEGF.

Materials and methods

Seventy-two adult male Sprague–Dawley rats weighing between 380 and 420 g were used in the experiment. The National Research Council's guidelines for the care and use of laboratory animals were followed. The rats were anaesthetised using pentobarbital administered by intraperitoneal injection (50 mg/kg). All surgical procedures were performed under sterile conditions. The 165 amino acid isoform of recombinant human VEGF (Genentech Inc., South San Francisco, CA, USA), suspended in phosphate-buffered saline (PBS), was used in this study.

Wound model

Simple incisional wound

Following induction of anaesthesia, a 3-cm linear full thickness incisional wound was made in the medial plane, beginning 1 cm below the inferior edge of scapula, through skin and panniculus carnosus. Haemostasis was obtained by direct pressure using sterile gauze. The wound was sutured with 4/0 nylon sutures in an interrupted fashion.

Ischaemic incision wound

A rat-back ischaemic wound model was established following the design from Quirinia.[19,20] An H-shaped double flap, consisting of a cranially based and a caudally based flap 2 cm wide and 4 cm long, was marked with ink. The ischaemic test wound was the horizontal bar in the H-shaped double flap. The skin and panniculus carnosus were incised. After the flaps were raised, perforating branches of the flaps were cut, and then the flaps were sutured back in position with 4/0 nylon sutures in an interrupted fashion.

Part I

Experimental design

Twenty-four rats were divided into two groups of 12 rats each. In group 1, 3-cm linear full thickness incision wounds were made below the inferior edge of scapula. In group 2, ischaemic wounds with the horizontal bar in the H-shaped double flap were made in rats. At 12 and 24 h ($n = 6$ for each interval) postoperatively, 4 mm full thickness skin punch biopsies were taken 0.5 cm from the center of the wound for VEGF protein examination (Fig. 1).

VEGF level determination

Tissue samples were homogenised in 400 μl PBS (pH 7.2). The homogenates were centrifuged at 15 000 rpm for 30 min at 4 °C. The supernatant was collected and stored at −80 °C until used. $VEGF_{165}$ was determined with an enzyme-linked immunosorbent assay (ELISA) kit (R & D systems, Minneapolis, MN, USA). Standards or samples (50 μl) were pipetted into each antibody-coated well containing 50 μl assay diluent and incubated for 2 h at room temperature. The wells were washed five times with wash buffer, and then 100 μl VEGF conjugate was added to each well. The samples were again incubated for 2 h at room temperature. After five washings, 100 μl substrate solution was added. Samples were incubated for 30 min at room temperature. Optical density was read in a plate reader (Bio-Tec Instruments, Inc., Winnoski, VT, USA) at the wavelength of 450 nm with a reference at 570 nm. The tissue sample concentration was calculated from the standard curve and normalised by the weight of the skin.

Part II

Experimental design

Forty-eight rats were divided into three groups with each group consisting of 16 rats.

In the first group, a simple incisional wound was made in each rat, and 1 ml saline was injected evenly into the subcutaneous tissue from both ends of the wound. In the second group, the ischaemic wound was established, and 1 ml recombinant human $VEGF_{-165}$ (1 μg/ml) was injected into the subcutaneous tissue at both sides of horizontal incision in the same manner as the first group. In the third group, the same amount of saline was injected into the subcutaneous tissue after establishment of the ischaemic wound (Fig. 2).

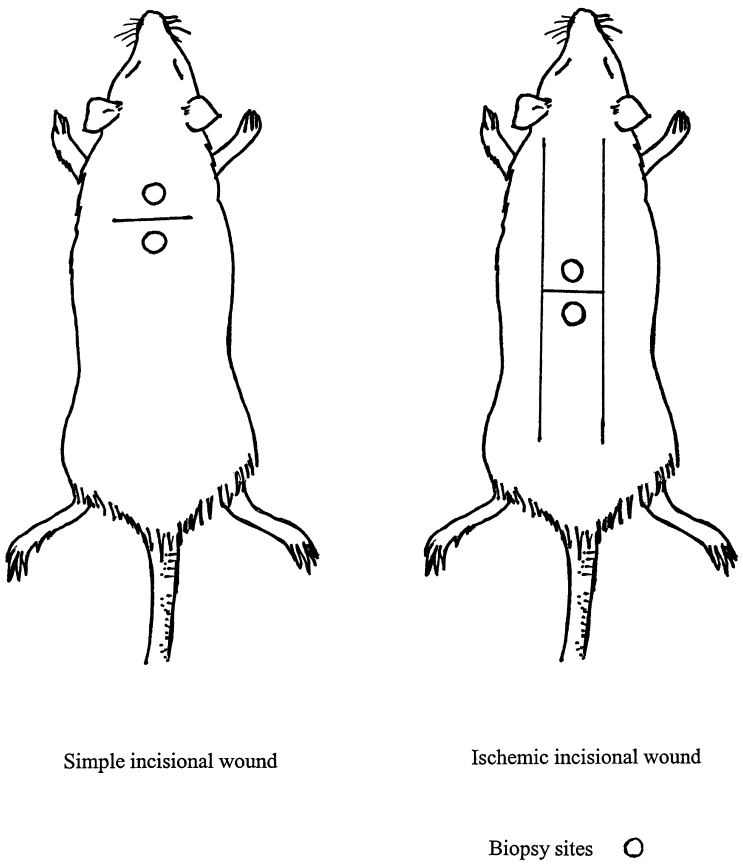


Fig. 1 Skin biopsies from the wounds.

Each group was divided into two subgroups according to the time of examination: 1 week and 2 weeks, postoperatively. At the examination time, the rats were reanaesthetised. A $2 \times 1\ cm^2$ skin sample was harvested from the center of the wound, including the horizontal incision, and prepared for tensile strength evaluation. The skin biopsies taken at one postoperative week were used for histologic study of angiogenesis.

Tensile strength test

The breaking strength of the repaired wound was tested using a skin tensiometer (Sintech 2/G, MTS, Minneapolis, MN, USA). The skin sample was placed vertically between two clamps of the tensiometer, and then subjected to a force applied with a 1-lb load cell across the incision with a constant speed of 10 mm/s until rupture. Applied maximum load was recognised as the maximum force and stress withheld before wound rupture. Tensile strength was calculated as the maximum breaking strength divided by cross-sectional area of the wound (MPa). Cross-sectional area was calculated as the product of the area subjected to testing between the clamps and the width of the specimen being tested.

CD31 immunohistochemical staining

The skin samples were fixed in 10% formalin solution and routinely processed for paraffin embedding. The sections (3–4 μm thick) were deparaffinised in xylene and hydrated through a graded series of ethanol, and then treated with 10% horse serum and 0.3% triton in 0.01 M PBS for 1 h at 37 °C. After being incubated in 0.3% H_2O_2 in methanol for 30 min and then rinsed in PBS, the slides were incubated with primary antibody (mouse anti-rat CD31, 1:100, Vector Lab, Inc., Purchase, NY, USA) with 10% horse serum at 4 °C overnight. The slides were incubated with biotimylated anti-mouse IgG (1:1000) for 1 h, and then incubated with abidin–biotin-complex for 30 min. After washing in PBS, the slides were developed by immersion into 0.02 H_2O_2 and 0.05% diaminobenzidine tetrahydrochloride for 7 min. The slides were rinsed with water, counterstained with 0.5 methyl green, and were ready for microvessel density (MVD) assessment. Semi-quantitative assessment of MVD in the wound samples was performed to measure the number of capillaries per high power field ($\times 40$ magnification).

After tensile strength examination and tissue

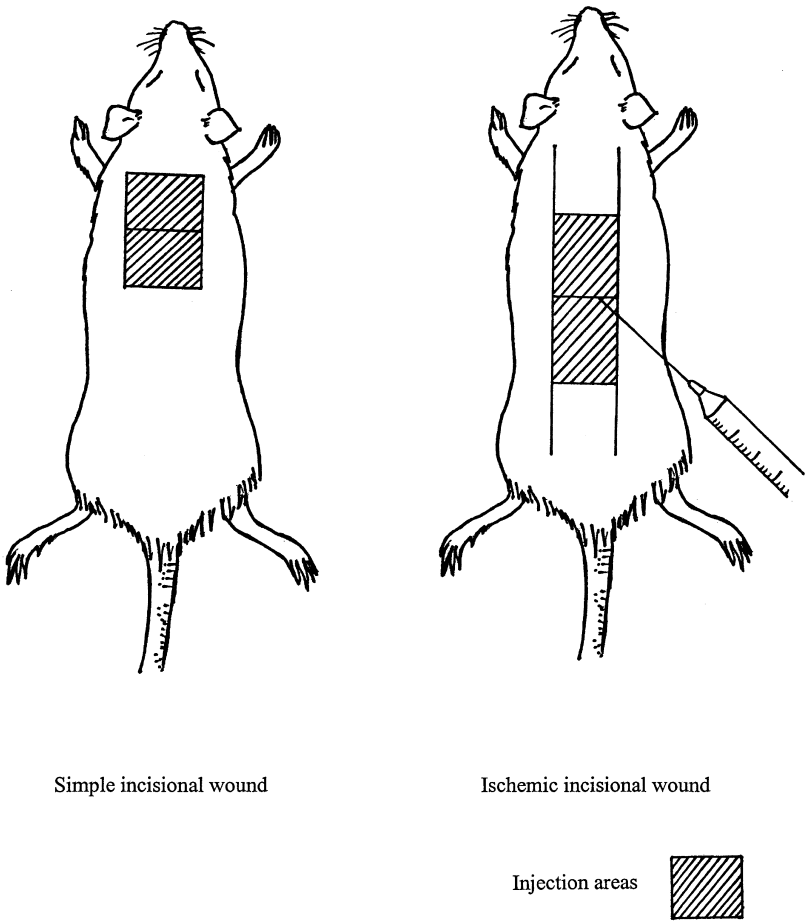


Fig. 2 The injection areas in treatment of wounds.

biopsies, the animals were sacrificed by an overdose pentobarbital. The data from the different groups were compared using analysis of variance (ANOVA) and Student's *t*-test. Statistical significance was assumed at $p < 0.05$.

Results

Part I

VEGF protein level

The values of VEGF protein from the different wound edges at each time interval are shown in Fig. 3. The VEGF level (mean ± SEM) in the simple incision wound was 0.18 ± 0.07 pg/mg tissue weight at 12 h, and slightly increased to 0.26 ± 0.11 pg/mg at 24 h postoperatively. In the ischaemic wound, the VEGF level was 1.04 ± 0.32 pg/mg at 12 h, and significantly increased to 2.43 ± 0.27 pg/mg at 24 h postoperatively. The difference in values between the simple incision wounds and ischaemic wounds was significant ($p < 0.01$).

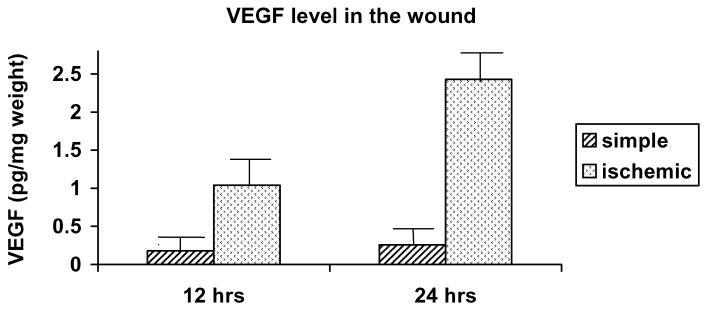
Part II

Gross examination

From gross examination, the surfaces of the simple incision and horizontal incision in the H-shaped double flaps healed without any complications in both experimental and control groups at 1 week and 2 weeks, postoperatively. The appearance, colour, and texture of the wound and double flaps were uniform in each group.

Mechanical test

The comparisons of the tensile strength (mean ± SD) of the repaired wound between different groups are shown in Table 1. At 1 week postoperatively, the tensile strengths were 0.59 ± 0.13 MPa in the simple incision wound repairs and 0.56 ± 0.09 MPa in the ischaemic wound repairs with VEGF treatment, which were significantly higher than the ischaemic wound repairs with saline injection (0.39 ± 0.04 MPa) ($p < 0.05$). At 2 weeks after surgery, there was no significant difference between the tensile strength in the simple incision wound repairs (0.57 ± 0.12 MPa) and the ischaemic



12 hrs. and 24 hrs.:

Ischemic wound vs. simple incision wound: $p<0.01$

Fig. 3 The comparison of results of VEGF protein level from the simple incisional wound and ischaemic wound.

wound repairs with VEGF (0.56 ± 0.05 MPa) and saline (0.52 ± 0.08 MPa) treatments ($p > 0.05$).

Immunohistochemical staining

The wound tissue samples from each experimental group at 1 postoperative week underwent MVD evaluation. All endothelial cells were stained with anti-rat CD31 antibody. Microvessels were represented by brown capillaries (Fig. 4). The MVD evaluation in the tissue samples was performed by measuring the number of capillaries per high power field. The semi-quantitative assessment of MVD (mean ± SD) showed a statistically significant difference between the simple incisional wound repair (2.1 ± 1.5 per field), ischaemic wound repair with VEGF treatment (5.8 ± 1.7 per field), and the ischaemic wound with saline treatment (1.9 ± 0.8 per field). There were no significant differences in MVD among the three groups at 2 weeks postoperatively. This comparison is shown in Fig. 5.

Table 1 Comparison of tensile strength test of wound healing (MPa, mean ± standard deviation)

Wound and treatment	Postoperative time intervals	
	1 Week postop	2 Weeks postop
Simple incision wound	0.59 ± 0.13	0.57 ± 0.12
Ischaemic wound with VEGF injection	0.56 ± 0.09	0.56 ± 0.05
Ischaemic wound with saline injection	0.39 ± 0.04	0.52 ± 0.08

1 Week postop: ischaemic wound with VEGF treatment vs. normal wound: $p > 0.05$; ischaemic wound with VEGF treatment vs. ischaemic wound with saline: $p < 0.05$. 2 Weeks postop: ischaemic wound with VEGF treatment vs. ischaemic wound with saline vs. normal wound: $p < 0.05$.

Discussion

Angiogenesis is a biological mechanism of new capillary formation and involves the activation, migration, and proliferation of endothelial cells from preexisting venules. Angiogenesis can be

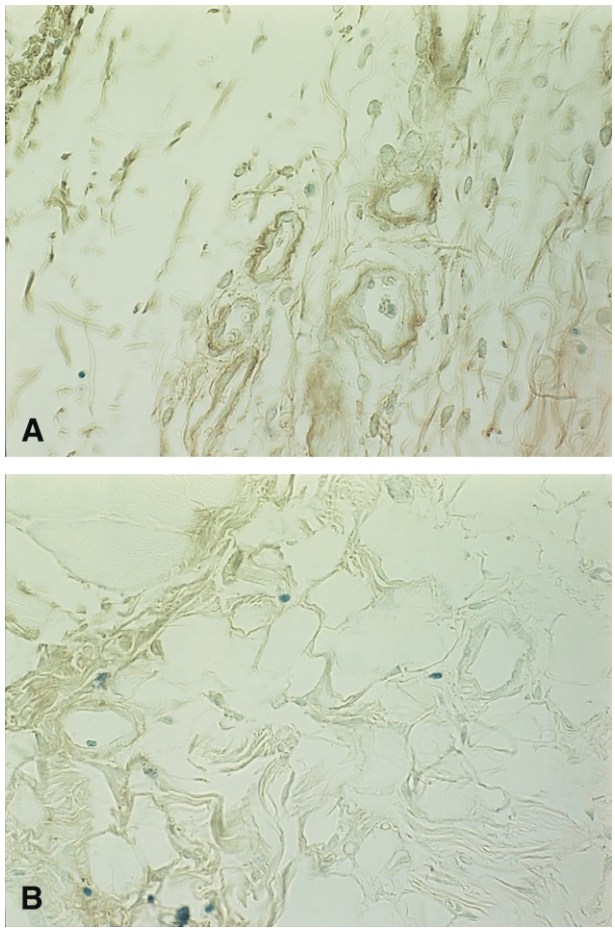


Fig. 4 The histologic anti-rat CD31 staining of wound tissue samples from: (A) ischaemic wound with VEGF injection; (B) ischaemic wound with saline injection, at 1 postoperative week. Endothelial cells stained with the antibody were represented by brown colour.

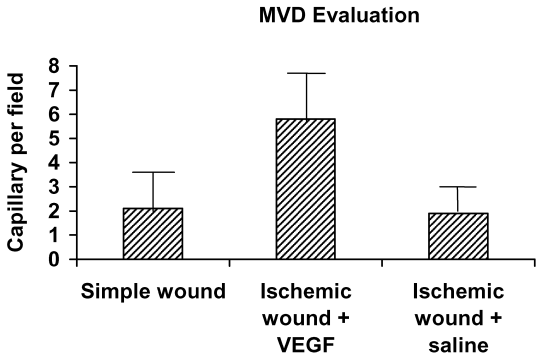


Fig. 5 The comparison of semi-quantitative assessment of MVD (mean ± SD) between each groups.

influenced by many factors including hypoxia, growth factors, matrix components, and metabolic gradients.[1-3] The angiogenic activation of endothelial cells probably plays a role in promoting and regulating other biological events, such as inflammation, fibroblast proliferation, extracellular matrix synthesis, and epithelialisation in wound healing.

VEGF is an endogenous stimulator of both angiogenesis and increased vascular permeability.[16-18] This process is believed to be essential for neovascularisation to occur. VEGF is expressed in developing blood vessels, and its receptors are found exclusively on endothelial cells.[21] The expression of VEGF is believed to be potentiated in response to ischaemia by activated oncogenes and a variety of cytokines.[22] VEGF has been demonstrated to mediate angiogenic activity during the proliferative phase of wound healing.[23] In an animal ischaemic wound healing model, ischaemia up-regulated VEGF mRNA expression 3- to 5-folds over nonischaemic skin. In contrast to VEGF, bFGF expression was not significantly induced by ischaemia; in fact there was a 2-fold reduction of bFGF mRNA levels in ischaemic skin.[24]

Recent evidence suggests that recombinant growth factor therapy may provide the added stimulus to the healing of certain types of chronic wounds.[8-15] In our preliminary studies, the administration of endogenous VEGF can significantly induce regional angiogenesis and improve the survival of random extension of axial pattern skin flaps.[28,29] Additionally, VEGF was found to accelerate the maturation of flap prefabrication, and accelerate the vascularisation of tubed pedicle flaps, which allows for earlier division of the tube.[30] It has also been found that VEGF appears to offer acute protection from ischaemia-reperfusion injury to a rat gracilis muscle flap model.[31] In other studies, a single intra-arterial or intravenous bolus of VEGF was shown to augment blood flow to collateral-dependent ischaemic heart muscle in a canine model.[32] Both intramuscular and intravenous administrations of VEGF increased blood flow to an ischaemic extremity. The animals treated with VEGF had higher calf systolic blood pressure ratios and higher capillary densities.[33] These findings provided us with the hypothesis that VEGF administration can promote ischaemic wound healing.

The wound created by H-shaped double flap model has been used to study ischaemic wound healing in the rat.[19,20,25-27] Prior studies have shown that blood flow in the H-shaped flap significantly decreased to 7% of the flow in the incisional wounds on postoperative day 1.[19] The reduction in blood flow resulted in a significant decrease in the biomechanical properties and thereby caused a significant delay in the healing of these ischaemic H-shaped wounds.[20] In the first part of the our study on the establishment of wound model, the observations on wound healing delay correlated with other investigators. These included significant loss of tensile strength in the wound created by H-shaped double flap, and up-regulation of VEGF expression in the tissues, which may be induced by ischaemia.

In the second part of the study, we evaluated the effect of exogenous VEGF on ischaemic wound healing. When the ischaemic incisional wound was treated with the exogenous VEGF, the tensile strength of the wound was significantly increased. This result indicated improvement of extracellular matrix synthesis in wound healing since the tensile strength is correlated with the concentration of collagen fibrils and pyridinoline collagen cross-links.[34,35] Histologically, the semi-quantitative level of MVD demonstrated that exogenous application of VEGF could significantly increase early angiogenesis in the wound, which in turns provides an environment conducive to wound healing.

In conclusion, the level of VEGF protein was

found to significantly increase in the ischaemic wound model compared to the incisional wound. However, the endogenous VEGF is apparently not enough for enhancement of early wound healing in the ischaemic wound. Application of exogenous VEGF could improve angiogenesis and tensile strength in the ischaemic wound, which possibly provide evidence for an angiomodulatory strategy VEGF plays in complicated wound treatment.

Key points

- What is VEGF and why is it important?
  VEGF is a potent direct angiogenic factor that has been shown to stimulate in vitro endothelial cell migration and activation, as well as in vivo angiogenesis. VEGF is expressed in developing blood vessels, and its receptors are found exclusively on endothelial cells.
- What do we already know about VEGF and wound healing?
  VEGF has been demonstrated to mediate angiogenic activity during the proliferative phase of wound healing. Ischaemia has been shown to increase the expression of VEGF over nonischaemic condition.
- What do we learn from this present study that is new?
  Ischaemic incisional wound treated with exogenous VEGF demonstrated a significant increase in mechanic property and angiogenesis.

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