Acute local subcutaneous VEGF\textsubscript{165} injection for augmentation of skin flap viability: efficacy and mechanism

Asim Khan,\textsuperscript{1,3} Homa Ashrafpour,\textsuperscript{1} Ning Huang,\textsuperscript{1} Peter C. Veligan,\textsuperscript{1,2} Christopher Kontos,\textsuperscript{4} Anguo Zhong,\textsuperscript{1} Christopher R. Forrest,\textsuperscript{1,2} and Cho Y. Pang\textsuperscript{1,2,3}

Departments of\textsuperscript{2}Surgery and \textsuperscript{3}Physiology, \textsuperscript{1}Research Institute, The Hospital for Sick Children, University of Toronto, Toronto, Ontario, Canada M5G 1X8; and \textsuperscript{4}Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710

Submitted 3 March 2004; accepted in final form 16 June 2004

Khan, Asim, Homa Ashrafpour, Ning Huang, Peter C. Veligan, Christopher Kontos, Anguo Zhong, Christopher R. Forrest, and Cho Y. Pang. Acute local subcutaneous VEGF\textsubscript{165} injection for augmentation of skin flap viability: efficacy and mechanism. Am J Physiol Regul Integr Comp Physiol 287: R1219–R1229, 2004. First published June 24, 2004; doi:10.1152/ajpregu.00143.2004.—Distal skin ischemic necrosis is a common complication in skin flap surgery. The pathogenesis of skin flap ischemic necrosis is unclear, and there is no clinical treatment available. Here, we used the 4 × 10 cm rat dorsal skin flap model to test our hypothesis that subcutaneous injection of vascular endothelial growth factor 165 (VEGF\textsubscript{165}) in skin flaps at the time of surgery is effective in augmentation of skin flap viability, which is associated with an increase in nitric oxide (NO) production, and the mechanism involves (1) an increase in skin flap blood flow in the early stage after surgery and (2) enhanced angiogenesis subsequently to sustain increased skin flap blood flow and viability. We observed that subcutaneous injection of VEGF\textsubscript{165} in skin flaps at the time of surgery increased skin flap viability in a dose-dependent manner. Subcutaneous injection of VEGF\textsubscript{165} at the dose of 2 \mu g/flap increased skin flap viability by 28\% (P < 0.05; n = 8). Over 80\% of this effect was blocked by intramuscular injection of the NO synthase (NOS) inhibitor \textsuperscript{N}\textsuperscript{O}-nitro-l-arginine (13 mg/kg) 45 min before surgery (P < 0.05; n = 8). The VEGF\textsubscript{165} treatment also increased skin flap blood flow (2.68 ± 0.63 ml·min\textsuperscript{-1}·100 g\textsuperscript{-1}; P < 0.05, n = 6) assessed 6 h postoperatively. There was no change in skin flap capillary density at this time point. VEGF\textsubscript{165}-induced increase in capillary density (32.2 ± 1.1 capillaries/mm\textsuperscript{2}; P < 0.05, n = 7) compared with control (24.6 ± 1.4 capillaries/mm\textsuperscript{2}) was seen 7 days postoperatively. There was also evidence to indicate that VEGF\textsubscript{165}-induced NO production in skin flaps was stimulated by activation of NOS activity followed by upregulation of NOS protein expression. These observations support our hypothesis and for the first time provide an important insight into the mechanism of acute local VEGF\textsubscript{165} protein therapy in mitigation of skin flap ischemic necrosis.

rat skin flaps; ischemic necrosis; nitric oxide; vasorelaxation; angiogenesis; vascular endothelial growth factor
VEGF165 in pig skin vasculature was predominantly mediated by the endothelium-derived relaxing factor nitric oxide (NO) (2). We believe that this vasodilator effect of VEGF165 plays a pivotal role in the efficacy of acute local VEGF165 protein or gene therapy for augmentation of skin flap viability. Therefore, we hypothesize that acute local VEGF165 protein therapy is effective in augmentation of skin flap viability associated with an increase in NO production, and the mechanism involves 1) an increase in skin flap blood flow in the early stage after surgery and 2) an enhanced angiogenesis subsequently to sustain increased skin flap blood flow and viability. We tested our hypothesis by studying the efficacy and mechanism of acute subcutaneous injection of VEGF165 in rat dorsal skin flaps for augmentation of skin flap viability. This rat dorsal skin model was also used by other investigators to investigate the efficacy of acute local VEGF165 therapy for augmentation of skin flap viability (15, 39). In addition, we previously used this skin flap model to study the pathophysiology of skin flap ischemic necrosis (5, 6).

MATERIALS AND METHODS

Animal Management

Male Sprague-Dawley rats were used for all studies. These rats were kept in individual cages in a temperature-controlled (22 °C) and light-controlled (0700–1900) animal holding room. All rats were offered the same commercial diet and tap water ad libitum. The mean body weight of these rats at the time of surgery was 386 ± 22 g (mean ± SD). The following surgical manipulation and experimental protocols were approved by the Animal Care Committee of The Hospital for Sick Children and were in compliance with the guidelines of the Canadian Council of Animal Care.

Experimental Surgery

Anesthesia. The rats were under general anesthesia during preparative operation, skin flap surgery, measurement of skin flap blood flow, assessment of skin flap viability, and taking biopsies and skin samples for histological and biochemical analysis, respectively. All procedures were performed in a temperature-controlled (24 °C) surgical room. General anesthesia was induced by intramuscular ketamine (90 mg/kg) and intraperitoneal pentobarbital sodium (20 mg/kg).

Skin flap surgery. The design and surgical technique for construction of the rat dorsal skin flap model for the study of skin flap distal ischemic necrosis and its prevention were described in detail previously (5, 6, 19). Briefly, a 4 × 10 cm caudally based acute random-pattern skin flap was raised on the dorsum of the rat. The skin flap was sutured to its bed with 3-0 silk sutures. After surgery, the rat was allowed to wake up and was returned to its cage in the animal holding room. The necrotic area was well demarcated in the distal portion of the skin flap and could be identified easily by gross observation 7 days postoperatively (19). The area of nonviable and viable skin in the skin flap was assessed using the template technique as previously described (5, 6, 31).

Acute subcutaneous injection of VEGF165 in rat dorsal skin flaps. The 165-amino acid isoform of recombinant human VEGF165 was donated by Genentech (San Francisco, CA). VEGF165 stock solutions were made with phosphate-buffered saline (pH 7.4) and were stored at −80 °C. Fresh VEGF165 solution in saline was made on the day of surgery. It was stored at 4 °C and used within 2 h. Immediately after raising of a 4 × 10 cm dorsal skin flap, 1 ml of saline with or without VEGF165 was drawn into a 1-ml syringe fitted with a 30-gauge needle. The saline or saline containing various concentrations of VEGF165 was injected subcutaneously in the rat dorsal skin flap through the panniculus carnosus along both sides of the midline at 1 cm from the midline. The injections were spaced 1.5 cm apart from the pedicle to the distal end of the skin flap. The skin flap was sutured to its bed immediately after subcutaneous injection. The rat was allowed to wake up and returned to its cage.

Collection of skin samples for biochemical analysis. Each 4 × 10 cm dorsal skin flap was cut into two halves with a pair of scissors along the longitudinal midline of the skin flap. Each half of the skin flap was then divided into four 2 × 2.5 cm sections from the pedicle to the distal end of the skin flap. This skin-collection technique allowed us to compare skin contents of the vasorelaxing factor NO, NOS enzyme (NOS) activities, and NOS protein expression in skin tissue at 0- to 2.5-, 2.5- to 5.0-, 5.0- to 7.5-, and 7.5- to 10.0-cm intervals from the pedicle of the skin flap. All skin samples were immediately rinsed with cold saline (4 °C), frozen in liquid nitrogen, and stored at −80 °C.

Biochemicals. Unless otherwise stated, all reagents and drugs were purchased from Sigma (Oakville, Ontario, Canada). Purified water (Milli-Q Water System, Bedford, MA) was used for making all solutions and buffers.

Analysis of capillary density. Permanent histological slide sections of 5 μm in thickness were prepared from paraffin-embedded skin tissue of full-thickness skin biopsies. All sections were stained with hematoxylin and eosin, and immunohistochemical technique was used for an immunohistochemical marker VIII-related antigen on the endothelial surface of skin vasculature as reported previously (13, 30). All slides were prepared by the Department of Histology at The Toronto General Hospital. Stained sections were viewed by a single observer, who was blinded to treatment regimen. Under ×250 magnification in a Leitz Orthoplan microscope (Leitz, Wetzlar, Germany), capillaries were identified by their single layer of flattened endothelial cells with factor VIII-related antigen immunostaining and the absence of a smooth muscle layer. In each slide section, capillaries were counted in eight random fields of 0.46 mm² each. Capillary density was calculated as number of capillaries per square millimeter field.

Measurement of total NO in skin samples. The end products of NO (NOX) are NO₂/NO₃⁻. The method for assessment of tissue contents of NOX was reported previously (11, 37). Briefly, frozen skin samples were crushed into small fragments, which were homogenized at 4 °C in a buffer (1 g/10 ml) containing (in mM) 25 Tris/HCl (pH 7.5), 0.5 EDTA, and 0.5 EGTA and centrifuged at 14,000 g at 4 °C for 15 min. The resulting supernatants were collected as cytosolic fractions for assay of protein content using the Bradford protein assay technique (Bio-Rad, Hercules, CA), and NOX content using the following technique (R&D Assay System, Minneapolis, MN). The supernatants were loaded to a centycon YM-30 filter (Millipore, Bedford, MA) and centrifuged at 7,000 g and 4 °C for 1.5 h to remove substances larger than 30 kDa. Nitrite was assayed using the Griess reaction. Nitrate content was determined after conversion of nitrate to nitrite with Aspergillus nitrate reductase. The skin contents of NOX were expressed as nmol/mg protein.

Measurement of NOS activity in skin samples. The methods for preparation of cytosolic and membrane fractions and for assessment of NOS activity in combined cytosolic and membrane fractions were similar to those described previously (33, 37). Briefly, frozen skin samples were crushed into small fragments, which were homogenized at 4 °C in a buffer (1 g/10 ml) containing 25 mM Tris/HCl (pH 7.4), 1 mM EGTA, 1 mM EDTA, 1% (vol/vol) Nonident P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, 1 μM pepstatin, and 1 μM aprotinin. The homogenates were centrifuged at 14,000 g at 4 °C for 20 min. The resulting supernatants were collected for measurement of protein content using the Bio-Rad DC protein assay technique (Bio-Rad, Hercules, CA) and assessment of NOS activity by measuring the conversion of L-[¹⁴C]arginine to L-[¹⁴C]citrulline. Briefly, 120 μl of 1 mg/ml protein were incubated for 60 min at 37 °C in 100 μl of assay buffer containing 50 mM Tris/HCl (pH 7.4), 10 μM L-arginine, 1 mM freshly made NaDPH, 5 μM flavin adenine dinucleotide, 5 μM flavin adenine mononucleotide, 10 μM tetrahydrobiopterin, 0.1 μCi
Protocol 2: investigation of the role of NO and cyclooxygenase products in acute local subcutaneous injection of VEGF165 for augmentation of skin flap viability in rat dorsal skin flaps. The inhibitory effects of the NOS inhibitor L-NNA and the cyclooxygenase inhibitor indomethacin were investigated in acute subcutaneous injection of VEGF165 in the rat dorsal skin flaps for augmentation of skin flap viability. Rats with a 4 × 10 cm dorsal skin flap were assigned to six groups with the following treatments: subcutaneous injection of 1 ml of saline in the rat dorsal skin flap immediately after raising of the skin flap (group 1); subcutaneous injection of 1 ml of saline containing 2 μg of VEGF165 in the rat dorsal skin flap immediately after raising of the skin flap (group 2); intramuscular injection of L-NNA (13 mg/kg) at 45 min before flap surgery and subcutaneous injection of 1 ml of saline containing 2 μg of VEGF165 in the rat dorsal skin flap immediately after raising of the skin flap (group 3); intramuscular injection of indomethacin (5 mg/kg) at 45 min before surgery and subcutaneous injection of 1 ml of saline containing 2 μg of VEGF165 immediately after raising of the skin flap (group 4); intramuscular injection of L-NNA (13 mg/kg) at 45 min before flap surgery (group 5); and intramuscular injection of indomethacin (5 mg/kg) at 45 min before flap surgery (group 6). All rats were allowed to wake up and were returned to their cages. Areas of viable and nonviable skin in the skin flap were assessed 7 days postoperatively.

Experimental Protocol

The following studies were designed to investigate whether acute subcutaneous injection of VEGF165 in rat dorsal skin flaps is effective in augmentation of skin flap viability and, if so, what mechanisms are involved in mediating VEGF165-induced increase in skin flap viability. The rats were killed with an overdose of pentobarbital sodium at the end of each experiment.

Protocol 1: investigation of the efficacy of acute local subcutaneous injection of VEGF165 for augmentation of skin flap viability in rat dorsal skin flaps. Saline (1 ml) or saline containing 1, 2, 4, or 20 μg of VEGF165 was drawn into a 1-ml syringe fitted with a 30-gauge needle. The saline or saline containing VEGF165 was injected subcutaneously in the rat dorsal skin flap immediately after the skin flap was raised, and the flap was sutured. The skin flap was sutured to its bed, and the rats were allowed to wake up and then returned to its cage. The areas of viable and nonviable skin in the skin flap were assessed 7 days postoperatively.
RESULTS

Efficacy of Acute Local Subcutaneous Injection of VEGF165 for Augmentation of Skin Viability in Rat Dorsal Skin Flaps

In this 4 × 10 cm caudally based rat dorsal skin flap model, it was well established that skin ischemic necrosis occurred only in the distal portion of the skin flaps. Subcutaneous injection of VEGF165 in rat dorsal skin flaps immediately after raising of skin flaps increased skin flap viability in a dose-dependent manner, with a maximum increase of 28% compared with the control at the dose of 2 μg/flap (Fig. 1). This dose of VEGF165 was used in the following studies because it resulted in a maximal effect in augmentation of skin flap viability. A 28% increase in skin flap viability would be a very significant improvement in clinical flap surgery.

Role of NO and Cylooxygenase Products in Acute Local Subcutaneous Injection of VEGF165 for Augmentation of Skin Flap Viability in Rat Dorsal Skin Flaps

Subcutaneous injection of VEGF165 at the time of surgery increased the skin flap viability compared with the control (P < 0.05; n = 8) (Fig. 2). Intramuscular injection of the NOS inhibitor L-NNA (13 mg/kg) at 45 min before surgery nearly completely (82%) blocked the increase in skin flap viability induced by acute local subcutaneous injection of VEGF165 (P < 0.05; n = 8). However, intramuscular injection of the cyclooxygenase inhibitor indomethacin (5 mg/kg) at 45 min before surgery had no significant inhibitory effect on the increase in skin flap viability induced by subcutaneous injection of VEGF165 at the time of surgery. The mean values of skin flap viability in rats injected intramuscularly with L-NNA or indomethacin alone at 45 min before surgery were similar to that of the control group (Fig. 2).

Effect of Acute Local Subcutaneous Injection of VEGF165 on Skin Blood Flow in Rat Dorsal Skin Flaps

The rat dorsal skin flaps injected subcutaneously with saline or saline containing VEGF165 (2 μg/flap) were similar in wet weight assessed 6 h postoperatively (Fig. 3). At this time point, the skin blood flow in the VEGF165-injected skin flaps was...
higher \(P < 0.05; n = 6\) than that of saline-injected control skin flaps (Fig. 3). When normalized to skin tissue weight, the skin blood flow in the VEGF165-injected skin flap \(2.68 \pm 0.63 \text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}\) was also higher \(P < 0.05; n = 6\) than that of the saline-injected control \(1.26 \pm 0.10 \text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}\). The mean arterial blood pressure measured during injection of radioactive microspheres for assessment of skin blood flow was similar between treatment rats and up to 9 cm from the pedicle in all six treatment rats and up to 5 cm from the pedicle in five of six treatment rats. Within control groups, the capillary density was higher \(P < 0.05; n = 7\) in biopsies taken 7 days \(24.6 \pm 1.4 \text{capillaries/mm}^2\) than 9 h \(20 \pm 0.6 \text{capillaries/mm}^2\) postoperatively. Similarly, within the treatment group, the capillary density was higher \(P < 0.05; n = 7\) in biopsies taken 7 days \(32.2 \pm 1.1 \text{capillaries/mm}^2\) than 9 h \(19.8 \pm 0.8 \text{capillaries/mm}^2\) postoperatively (Fig. 6).

Of particular importance was the observation that the capillary density was similar between control and treatment skin biopsies taken 9 h postoperatively (Fig. 6). However, the capillary density in treatment skin biopsies was higher \(P < 0.05; n = 7\) than that of the control skin biopsies taken 7 days postoperatively.

**Effect of Acute Local Subcutaneous Injection of VEGF165 on Skin Contents of NOx in Rat Dorsal Skin Flap**

Subcutaneous injection of VEGF165 \(2 \mu g/\text{flap}\) at the time of surgery caused a significant \(P < 0.05; n = 7\) increase of skin content of NOx along the entire length of rat dorsal skin flaps at 9 h postoperatively compared with the time-matched control (Fig. 7A). Subcutaneous injection of VEGF165 at the time of surgery also increases NOx content along the entire length of rat dorsal skin flaps at 24 h postoperatively, and a statistical significant difference \(P < 0.05; n = 7\) in the skin content of NOx between control and treatment skin flaps was detected from 5.0–10.0 cm from the pedicle compared with the time-matched control (Fig. 7B).

**Effect of Acute Local Subcutaneous Injection of VEGF165 on Skin Tissue NOS Activity and Protein Expression**

Subcutaneous injection of VEGF165 in rat dorsal skin flaps at the time of surgery induced a significant \(P < 0.05\) increase in cNOS activities along the entire length of the skin flap compared with the saline-injected control skin flaps assessed 9 h postoperatively (Fig. 8A). However, there were no differences in cNOS activity between control and treatment skin flaps assessed at 24 h postoperatively (Fig. 8B). Skin iNOS activity was similar between control and treatment dorsal skin flaps assessed at 9 h (Fig. 9A) and 24 h (Fig. 9B) postoperatively.

Western blot analysis was used to study eNOS protein expression in skin samples. In our preliminary experiment, equal protein loading technique for Western blot analysis was confirmed by visualization of total protein on loaded gels using Coomassie brilliant blue and Western blots probed with an anti B-actin antibody (Fig. 10).

The skin protein levels of eNOS assessed 9 h after subcutaneous injection of VEGF165 in rat dorsal skin flaps were significantly \(P < 0.05; n = 7\) higher than those of the time-matched control along the entire length of the skin flaps (Fig. 11). Similarly, the skin protein levels of eNOS assessed 24 h after subcutaneous injection of VEGF165 in rat dorsal skin flaps were higher than those of the time-matched control along the entire length of the skin flap (Fig. 12). Statistical significance in skin protein levels of eNOS between control and treatment skin flaps was detected up to 5 cm from the pedicle and 7.5–10.0 cm from the pedicle \(P < 0.05; n = 9\), whereas differences in skin protein levels of eNOS from 5.0 to 7.5 cm from the pedicle boarded on statistical significance \(P < 0.07\).
DISCUSSION

Major Findings in the Present Studies

Using the rat dorsal skin flap model, we have investigated for the first time the mechanism of action of acute local subcutaneous injection of VEGF165 on skin flap blood flow and viability. Specifically, we have observed for the first time that subcutaneous injection of VEGF165 in skin flaps at the time of surgery increased skin flap viability in a dose-dependent manner. Subcutaneous injection of VEGF165 at the dose of 2 μg/flap increased skin flap viability by 28% (P < 0.05; n = 8). Over 80% of this effect on skin flap viability was blocked by intramuscular injection of the NOS inhibitor L-NNA (13 mg/kg) 45 min before surgery. This VEGF165 treatment also increased skin flap blood flow assessed 6 h postoperatively (P < 0.05; n = 6), but there was no change in capillary density at this time point. A VEGF165-induced increase in capillary density was seen 7 days postoperatively. There was also evidence to indicate that VEGF165-induced NO production was stimulated by activation of eNOS activity followed by upregulation of eNOS protein expression. These observations led us to conclude that local subcutaneous injection of VEGF165 in skin flaps at the time of surgery is effective in augmentation of skin flap viability by an increase in NO production, and the mechanism involves the vasodilator effect of VEGF165 in the early stage followed by the angiogenic effect of VEGF165 in late stage after surgery.

Efficacy of Acute Local Subdermal/Subcutaneous VEGF165 Protein Therapy in Augmentation of Skin Flap Viability

It was observed that subdermal injection of VEGF165 protein in rat dorsal skin flaps at the time of surgery attenuated skin flap ischemic necrosis (15, 39). In the present study, we demonstrated for the first time that subcutaneous injection of VEGF165 protein in rat dorsal skin flaps caused an increase in skin flap viability in a dose-dependent manner (Fig. 1). On the other hand, it was reported recently that injection of VEGF165 into the panniculus carnosus of rat abdominal arterial skin flaps at the time of surgery did not increase skin flap viability (16). In that study, the dose of VEGF165 used was 1 μg/skin flap. Although this dose of VEGF165 increased skin flap viability by 24% compared with the control, a statistical significance was not achieved. It is possible that a significant increase in skin flap viability would have been achieved if a higher dose of...
VEGF 165 were used in that study. According to our present data on the dose-response effect of subcutaneous injection of VEGF 165 in augmentation of skin flap viability, the effective dose of VEGF 165 required to induce a significant increase in skin viability in that size of rat skin flap would be \( 2/\text{H}9262 \) g/skin flap (Fig. 1). Taken together, the experimental evidence available thus far seems to indicate that subdermal or subcutaneous injection of VEGF165 in rat skin flaps given at the time of surgery effectively augments skin flap viability.

**Role of Vasodilator and Angiogenic Effect of VEGF 165 in Augmentation of Skin Flap Viability**

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**Group 1 - Skin Flap Viability**

Fig. 6. Capillary density in skin biopsies taken from rat dorsal skin flaps at 9 h or 7 days after surgery. Control and treatment skin flaps received subcutaneous injection of saline (1 ml) and saline containing 2 \( \mu \)g of VEGF165, respectively, immediately after skin flaps were raised. Values are means ± SE; \( n = 7 \). *Significantly different from corresponding control (\( P < 0.05 \); 2-way ANOVA and Neuman-Keuls multiple comparison test).

**Group 2 - Nitric Oxide (NO) Content**

Fig. 7. Skin content of total nitric oxide (NOx) in rat dorsal skin flaps at 9 h and 7 days after surgery. Values are means ± SE; \( n = 7 \). *Significantly different from corresponding control (\( P < 0.05 \); 2-way ANOVA and Neuman-Keuls multiple comparison test).

**Group 3 - Calcium-dependent Nitric Oxide Synthase (cNOS) Activity**

Fig. 8. Calcium-dependent NOS (cNOS) activity in rat dorsal skin flaps at 9 h and 24 h after surgery. Values are means ± SE; \( n = 7 \). *Significantly different from the corresponding control (\( P < 0.05 \); 2-way ANOVA and Neuman-Keuls multiple comparison test).

Fig. 7. Skin content of total nitric oxide (NOx) in rat dorsal skin flaps at 9 h and 7 days after surgery. Values are means ± SE; \( n = 7 \). *Significantly different from corresponding control (\( P < 0.05 \); 2-way ANOVA and Neuman-Keuls multiple comparison test).

Fig. 8. Calcium-dependent NOS (cNOS) activity in rat dorsal skin flaps at 9 h and 24 h after surgery. Values are means ± SE; \( n = 7 \). *Significantly different from the corresponding control (\( P < 0.05 \); 2-way ANOVA and Neuman-Keuls multiple comparison test).
VEGF 165 protein or gene therapy given 12 h before or at the time of surgery as discussed above. Consequently, it is most likely that another mechanism is also involved. Recently, we have demonstrated that VEGF 165 is a potent vasodilator in isolated perfused pig skin flaps in vitro (2). In the present studies, we also observed that the vasodilator effect of VEGF<sub>165</sub> protein or gene therapy given 12 h before or at the time of surgery as discussed above. Consequently, it is most likely that another mechanism is also involved.

Fig. 11. Endothelial NOS (eNOS) protein expression in rat dorsal skin flaps 9 h after subcutaneous injection of saline (1 ml) or saline containing 2 μg of VEGF<sub>165</sub>. Top: representative autoradiographs. Bottom: eNOS protein levels quantified by scanning densitometry and normalized to the control. Results are expressed as a percentage of the corresponding control for skin samples obtained for 0–2.5, 2.5–5.0, 5.0–7.5, and 7.5–10.0 cm from the pedicle. Values are means ± SE; n = 8. *Significantly different (P < 0.05) from corresponding control.

Fig. 12. eNOS protein expression in rat dorsal skin flaps 24 h after subcutaneous injection of saline (1 ml) or saline containing 2 μg of VEGF<sub>165</sub>. Top: representative autoradiographs. Bottom: eNOS protein levels quantified by scanning densitometry and normalized to the control. Results are expressed as a percentage of the corresponding control for skin samples obtained from 0–2.5, 2.5–5.0, 5.0–7.5, and 7.5–10.0 cm from the pedicle. Values are means ± SE; n = 9. *Significantly different (P < 0.05) from corresponding control.
VEGF<sub>165</sub> also plays a central role in the VEGF<sub>165</sub>-induced increase in skin flap viability. Specifically, subcutaneous injection of VEGF<sub>165</sub> in rat dorsal skin flaps at the time of surgery increased skin flap viability (Fig. 1) and blood flow, especially in the distal portion of the skin flap, compared with the control (Figs. 3 and 4). This increase in skin flap blood flow assessed at 6 h postoperatively was not associated with an increase in capillary density, i.e., angiogenesis (Fig. 6). However, enhanced angiogenesis was observed 7 days postoperatively in rat dorsal skin flaps injected subcutaneously with VEGF<sub>165</sub> at the time of surgery compared with the saline-injected control (Fig. 6). It is important to point out that other investigators have also reported that angiogenesis was observed between 18 and 24 h after intradermal injection of VEGF<sub>165</sub> in the mouse (28). Taken together, these observations indicate that acute local subcutaneous injection of VEGF<sub>165</sub> induces two stages of action in augmentation of skin flap viability. In the early stage, the vasodilator effect of VEGF<sub>165</sub> most likely mitigates vasoconstriction caused by surgical trauma, and the angiogenic effect of VEGF<sub>165</sub> increases capillary density in the later stage to sustain skin flap blood flow and viability, especially in the distal portion of the skin flap.

**Role of NO and Cyclooxygenase Products in Mediation of VEGF<sub>165</sub>-Induced Increase in Skin Flap Viability**

In the present studies, we observed that intramuscular injection of the NOS inhibitor L-NNA (13 mg/kg) 45 min before surgery blocked the VEGF<sub>165</sub>-induced augmentation of skin flap viability by 87% ($P < 0.05; n = 8$). However, intramuscular injection of indomethacin (5 mg/kg) 45 min before flap surgery had no significant effect on VEGF<sub>165</sub>-induced increase in skin flap viability (Fig. 2), although this dose of indomethacin is known to be highly effective in inhibition of cyclooxygenase in the rat (24). Therefore, we speculate that NO plays the predominant role in mediating the VEGF<sub>165</sub>-induced increase in skin flap viability in the rat, but this mechanism is unclear. Using the isolated perfused pig buttck skin flap model, our laboratory previously demonstrated that VEGF<sub>165</sub> stimulated NO and prostacyclin synthesis in skin vasculature, but the NOS inhibitor L-NNA predominantly blocked the VEGF<sub>165</sub>-induced vasorelaxation (2). Other investigators also observed that the NOS inhibitor N<sup>ω</sup>-monomethyl-L-arginine blocked the VEGF<sub>165</sub>-induced migration and proliferation (i.e., angiogenesis) of human umbilical vein endothelial cells (34). Taken together, these observations indicate that L-NNA treatment in the present study may have blocked the VEGF<sub>165</sub>-induced vasorelaxation and angiogenesis mediated by NO, resulting in attenuation of VEGF<sub>165</sub>-induced increase in skin flap viability in the rat.

**Mechanism of VEGF<sub>165</sub>-induced NO Production in Rat Dorsal Skin Flaps for Augmentation of Skin Flap Blood Flow and Viability**

Using the isolated perfused pig buttck skin flap model, our laboratory has recently demonstrated that the vasorelaxation effect of VEGF<sub>165</sub> is mediated by VEGF receptor-2, and the postreceptor signal pathway involves activation of phospholipase C and protein kinase C, increase in inositol 1,4,5-triphosphate activity, release of intracellular Ca<sup>2+</sup> stores, and synthesis/release of NO (2). Ca<sup>2+</sup> is also known to activate NOS for NO synthesis (9). In the present study, the skin contents of NOx and eNOS activities along the entire length of the rat dorsal skin flap assessed 9 h postoperatively were higher ($P < 0.05; n = 7$) in skin flaps injected subcutaneously with VEGF<sub>165</sub> at the time of surgery than those of saline-injected controls (Figs. 7A and 8A). These observations led us to speculate that the increase in intracellular free Ca<sup>2+</sup> induced by VEGF<sub>165</sub> injection at the time of surgery stimulated the activity of eNOS for NO synthesis in the early stage after skin flap surgery. An increase in eNOS activity was not seen at 24 h postoperatively in skin flap injected subcutaneously with VEGF<sub>165</sub> compared with the saline-treated control.

However, we also observed that eNOS protein expression along the entire length of rat dorsal skin flaps assessed 9 and 24 h postoperatively was higher in the skin flaps injected subcutaneously with VEGF<sub>165</sub> at the time of surgery compared with saline-injected controls (Figs. 11 and 12). This observation indicated that upregulation of VEGF<sub>165</sub>-induced NOS protein expression in rat dorsal skin flaps also occurred within 9–24 h after VEGF<sub>165</sub> injection. Increases in eNOS protein expression at this period of time could have also contributed to the increase in NOx synthesis seen at 9 and 24 h postoperatively (Fig. 7). This pattern of VEGF<sub>165</sub>-induced upregulation of eNOS was also reported by other investigators. Specifically, it was reported that upregulation of eNOS gene expression started 6 h and peaked at 9 h after exposure of cultured human endothelial cells to VEGF<sub>165</sub> (10), and upregulation of eNOS gene and protein expression occurred in rat aortic segments at 6 and 24 h after exposure to VEGF<sub>165</sub> (3).

Other investigators observed that subcutaneous injection of VEGF<sub>165</sub> in rat dorsal skin flaps at the time of surgery did not increase skin iNOS gene expression at 12 and 24 h postoperatively (26). We also did not observe any change in iNOS activity in skin flaps at 9 and 24 h postoperatively (Fig. 9). Therefore, it is most likely that iNOS was not involved in the increase in NO production in skin flaps in the present studies.

**Effect of VEGF<sub>165</sub> on Skin Vascular Permeability**

Using the isolated perfused pig skin flap model, we demonstrated previously that intra-arterial infusion of VEGF<sub>165</sub> also increased vascular permeability resulting in ~5% water retention at the end of 2 h of in vitro perfusion (2). However, this small extent of edema did not affect the vasodilator effect of VEGF<sub>165</sub>. In the present studies, subcutaneous injection of VEGF<sub>165</sub> (2 µg/flap) in skin flaps did not seem to increase water retention because the mean wet weight of skin flaps injected with VEGF<sub>165</sub> was similar to that of the saline-injected controls 6 h after injection (Fig. 3). In addition, we observed in our preliminary experiments that the water content of rat dorsal skin flaps injected with 2 µg of VEGF<sub>165</sub> (74 ± 1%) was similar to that of the saline-injected controls (73 ± 1%; n = 4) at 24 h after VEGF<sub>165</sub> injection.

In summary, observations made from the present studies support our hypothesis that subcutaneous injection of VEGF<sub>165</sub> in rat dorsal skin flaps at the time of surgery effectively attenuates skin flap ischemic necrosis, mainly by inducing the synthesis/release of the vasorelaxing factor NO. The mechanism by which VEGF<sub>165</sub> prevents skin flap ischemic necrosis appears to depend on the vasodilator effect of VEGF<sub>165</sub> in the
early stage, followed by the angiogenic effect of VEGF_165 in the late stage after surgery.

Perspectives

Skin flaps are routinely used for coverage of large deep wounds/tissue defects. The number of cases of skin flap surgery will continue to increase because of a growing population of elderly citizens, and it is this group of patients who frequently have problems in wound healing, ulceration, and threatened ischemic limbs that require skin flap coverage in reconstructive surgery. The information obtained from the present studies provides insight into the development of a simple, safe, and inexpensive local prophylactic treatment modality for the prevention of skin flap ischemic necrosis in high-risk patients scheduled for skin flap surgery.

ACKNOWLEDGMENTS

The authors thank D. McIntyre for word processing in preparation of this manuscript.

GRANTS

This research project was supported by an operating grant (MOP 8048) from the Canadian Institutes of Health Research (to C. Y. Pang) and Wharton Endowment Fund (to P. C. Neligan).

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