Efficacy and mechanism of adenovirus-mediated VEGF-165 gene therapy for augmentation of skin flap viability

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Huang, Ning, Asim Khan, Homa Ashrafpour, Peter C. Neligan, Christopher R. Forrest, Christopher D. Kontos, and Cho Y. Pang. Efficacy and mechanism of adenovirus-mediated VEGF-165 gene therapy for augmentation of skin flap viability. Am J Physiol Heart Circ Physiol 291: H127–H137, 2006. First published February 3, 2006; doi:10.1152/ajpheart.01253.2005.—Skin ischemic necrosis due to vasospasm and/or insufficient vascularity is the most common complication in the distal portion of the skin flap in reconstructive surgery. This project was designed to test our hypothesis that preoperative subdermal injection of adenoviral vectors encoding genes for vascular endothelial growth factor-165 (Ad.VEGF-165) or endothelial nitric oxide (NO) synthase (Ad.eNOS) effectively augments skin viability in skin flap surgery and that the mechanism of Ad.VEGF-165 gene therapy involves an increase in synthesis/release of the angiogenic and vasodilator factor NO. PBS (0.5 ml) or PBS containing Ad.VEGF-165, Ad.eNOS, or adenovirus (Ad.Null) was injected subdermally into the distal half of a mapped rat dorsal skin flap (4 × 10 cm) 7 days preoperatively, and skin flap viability was assessed 7 days postoperatively. Local subdermal gene therapy with 2 × 107–2 × 1010 plaque-forming units of VEGF-165 increased skin flap viability compared with PBS- or Ad.Null-injected control (P < 0.05). Subdermal Ad.VEGF-165 and Ad.eNOS gene therapies were equally effective in increasing skin flap viability at 5 × 108 plaque-forming units. Subdermal Ad.VEGF-165 therapy was associated with upregulation of eNOS protein expression, Ca2+-dependent NO activity, synthesis/release of NO, and an increase in capillary density and blood flow in the distal portion of the skin flap. Injection of the NO inhibitor Nω-nitro-L-arginine (15 mg/kg im), but not the cyclooxygenase inhibitor indomethacin (5 mg/kg im), 45 min preoperatively completely abolished the increase in skin flap blood flow and viability induced by Ad.VEGF-165 injected subdermally into the mapped skin flap 7 days preoperatively. We have demonstrated for the first time that 1) Ad.VEGF-165 and Ad.eNOS mapped skin flap injected subdermally into the mapped skin flap 7 days preoperatively are equally effective in augmenting viability in the rat dorsal skin flap compared with control, 2) the mechanism of subdermal Ad.VEGF-165 gene therapy in augmenting skin flap viability involves an increase in NO synthesis/release downstream of upregulation of eNOS protein expression and Ca2+-dependent NOS activity, and 3) the vasodilating effect of NO may predominantly mediate subdermal Ad.VEGF gene therapy in augmenting skin flap blood flow and viability.

subdermal gene transfer; nitric oxide; capillary density; vasodilation; skin blood flow

IN RECONSTRUCTIVE SURGERY, skin flaps are frequently used to cover large deep wounds or tissue defects resulting from injury, excision of tumors, ulceration, and congenital malformation or for reconstruction of facial structures such as the ear, nose, lip, and forehead (6, 10). In skin flap surgery, a large piece of full-thickness skin with subcutaneous tissue is undermined from the donor site and immediately transferred and sutured to the nearby wound, with the pedicle of the skin flap remaining attached to the donor site for blood supply (i.e., pedicled skin flap). When the wound is at a distant site, the skin flap is detached from the donor site and transferred with its feeding blood vessels to the recipient site, where the dominant artery and vein of the skin flap are anastomosed to corresponding blood vessels in the recipient site for blood supply; this operation is known as autogenous skin transplantation or skin free-flap surgery (6, 10, 39). Distal skin ischemic necrosis is a well-known complication in skin flap surgery. The general consensus is that unpredictable vasospasm, thrombosis, and insufficient vascularity are the main factors in the pathogenesis of skin flap ischemic necrosis (6). Clinically and experimentally, the surgical delay procedure is the only proven technique to prevent skin flap ischemic necrosis. Specifically, a bipedicled skin flap is constructed on the donor site to induce local angiogenesis and/or vasodilation, probably due to mild hypoxia, and the bipedicled skin flap is converted to a single pedicled skin flap 2–3 wk later for wound coverage (10). However, this surgical delay procedure is not a practical solution. It is time consuming and costly, in that it requires several surgical procedures and a prolonged period of hospitalization, and there is always the risk of infection due to repeated surgery.

Pharmacological treatment to mimic the surgical delay phenomenon in augmenting skin flap viability has been the focus of much research in skin flap surgery in the past two decades. Numerous systemic and several topical vasodilator and systemic antithrombotic drugs have been investigated in laboratory animals for augmenting skin flap viability in pedicled skin flaps, and the results are controversial or modest at best, and none of the drugs have reached clinical use (6, 28). More recently, skin flap research has focused on the use of vascular endothelial growth factor (VEGF) to promote local angiogenesis and, in turn, augment skin flap blood flow and viability. For example, skin viability in rat musculocutaneous flaps was significantly increased by local subcutaneous injection of recombinant human VEGF-165 at 7 days before surgery (37). Local intra-arterial injection of recombinant human VEGF-165 along with 10 min of vascular bed incubation 7 days before surgery Augmentation of skin flap viability involving increased synthesis and release of NO involves an increase in capillary density and blood flow in the distal portion of the skin flap.
surgery was also effective in augmenting rat skin flap viability (36). It was speculated that angiogenesis plays an important role in VEGF-165 protein therapy in augmentation of skin flap viability (36, 37). However, other investigators reported that local subcutaneous or subdermal injection of VEGF-165 protein into rat skin flaps or musculocutaneous flaps at the time of surgery was also effective in augmenting skin viability (16, 19, 26, 33, 47, 48). In addition, we demonstrated that intra-arterial infusion of VEGF-165 induced potent relaxation in isolated perfused pig skin flaps (2) and that acute local subcutaneous injection of VEGF-165 increased skin blood flow and viability in rat dorsal skin flaps (16).

The biological half-life of VEGF-165 is ∼30–45 min in normoxa and 6–8 h in hypoxia (40). Because the effectiveness of VEGF-165 protein therapy might be limited by its short half-life, VEGF-165 gene therapy may be the key to a steady release of VEGF-165 perioperatively (43). Experimental evidence indicates that VEGF-165 gene therapy is feasible in augmenting skin flap viability. Specifically, local subdermal or subcutaneous injection of liposomal or adenoviral vectors encoding the cDNA of VEGF-165 at 0.5, 2, 3, 7, or 14 days before surgery effectively augmented skin flap viability in the rat, but the mechanism was not studied (9, 11, 20, 21). Local subcutaneous injection of VEGF-165 plasmid DNA 7 days preoperatively also increased skin viability in rat musculocutaneous flaps, and again the mechanism was not studied (49). Other investigators observed that local adenovirus-mediated VEGF-165 gene therapy induced angiogenesis in the skin of the mouse ear and in rat skin flaps (32, 46), but the mechanism was also not studied. An understanding of the efficacy and mechanism of adenovirus-mediated gene therapy in augmenting skin flap viability will provide important insight into the clinical application of this treatment modality in the future. Therefore, this research project was designed to test our hypothesis that preoperative local subdermal adenovirus-mediated VEGF-165 (Ad.VEGF-165) and endothelial nitric oxide (NO) synthase (Ad.eNOS) gene therapies are effective in augmenting skin flap viability and that the mechanism of Ad.VEGF-165 gene therapy involves an increase in synthesis/release of the angiogenic and vasodilator factor NO.

MATERIALS AND METHODS

Animal Management

Male Sprague-Dawley rats (350–375 g body wt) were housed in individual cages in a light-controlled (0700–1900) and temperature-controlled (22°C) animal holding room in an isolated rat containment unit under conditions outlined in the institutional guidelines for use of replication-deficient recombinant adenovirus vectors. All rats were offered the same commercial feed and tap water ad libitum. The 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. General anesthesia was induced by ketamine (90 mg/kg im) and pentobarbital sodium (~20 mg/kg ip). The rats were anesthetized during skin flap surgery, catheterization, measurement of skin flap blood flow, excision of skin samples for histological and biochemical analysis, and assessment of skin flap viability. At the end of each experiment, the rats were killed with an overdose of pentobarbital sodium (100 mg/kg ip).

Skin flap surgery. The design and surgical technique for construction of the rat dorsal random-pattern skin flaps were described previously (16, 19, 23, 46, 48). Briefly, a 4 × 10 cm caudally based skin flap was raised on the dorsum of the rat. The skin flap was returned and sutured to its bed with 3-0 silk sutures. After surgery, the rat was allowed to awaken and was returned to its cage in the animal holding room. At 7 days after skin flap surgery, the ischemic necrotic area was well demarcated in the distal portion of the skin flap; it could be identified easily by gross observation (23), and the area of viable and nonviable skin in the skin flap was assessed using the template technique described previously (16, 23, 35).

Adenovirus-Mediated VEGF-165 Gene Therapy

Adenovirus encoding the recombinant human VEGF-165 gene under control of a cytomegalovirus promoter (Ad.VEGF-165) and control adenovirus without the VEGF-165 gene (Ad.Null) were provided by Dr. Christopher Kontos (Duke University Medical Center). Ad.Null and Ad.VEGF-165 stock solutions were made with PBS (pH 7.4) and stored at −80°C. Fresh Ad.VEGF-165 solutions [2 × 10^10–2 × 10^11] plaque-forming units (pfu)] were made with PBS (0.5 ml) on the day of injection. The solutions were stored at 4°C and used within 2 h.

Because skin ischemic necrosis in the 4 × 10 cm rat dorsal skin flap model occurs in the distal half of the skin flap, i.e., 5–10 cm from the pedicle (8, 16, 23), gene therapy was administered in this area of the skin flap. Specifically, 0.5 ml of PBS or PBS containing Ad.Null or various concentrations of Ad.VEGF-165 was drawn into a 0.5-ml syringe fitted with a 30-gauge needle and injected subdermally into the distal half of the mapped skin flap 7 days before surgery. The injections were spaced 0.5 cm apart along both sides of the midline, at 1 cm from the midline.

Adenovirus-Mediated eNOS Gene Therapy

Adenoviral vector encoding the recombinant bovine eNOS gene under the control of a cytomegalovirus promoter (Ad.eNOS) and Ad.Null were donated by Dr. Zvonimir Katusic (Mayo Clinic, Rochester, MN). The experimental procedure for local subdermal Ad.eNOS gene therapy in the rat dorsal skin flap was similar to that for Ad.VEGF-165 (see Adenovirus-Mediated VEGF-165 Gene Therapy).

Collection of Skin Samples for Biochemical Assay

Each 4 × 10 cm dorsal skin flap was cut into two halves along the longitudinal midline. The skin segments 5.0–7.5 and 7.5–10.0 cm from the pedicle of the skin flap were cut and collected for biochemical analyses (see below). All skin samples were immediately rinsed with cold saline (4°C), frozen in liquid nitrogen, and stored at −80°C.

Because the critical time for ischemia of skin flaps in laboratory animals is 6–13 h (1, 15, 22, 24, 38, 44), skin samples were taken for study of capillary density and biochemical assay and measurement of skin flap blood flow were performed 9 h after skin flap surgery.

Measurement of Total NO in Skin Samples

Tissue contents of total end products of NO (NOx, NO2, and NO3) were measured as described previously (14, 41, 45). Briefly, frozen skin samples were crushed and homogenized at 4°C in a buffer (1 g/10 ml) containing 25 mmol/l Tris–HCl (pH 7.4), 0.5 mmol/l EDTA, and 0.5 mmol/l EGTA and centrifuged (14,000 g, 4°C) for 20 min. The resulting supernatants were collected for assay of protein content (Bradford protein assay, Bio-Rad, Hercules, CA) and NOx (R & D Assay System, Minneapolis, MN). The supernatants were loaded onto a filter (Centricon YM-30, Millipore, Bedford, MA) and centrifuged (7,000 g, 4°C) for 1.5 h to remove >30-kDa substances. NO2 content was assayed using the Griess reaction, and NO3 content was assayed...
after conversion of $\text{NO}_3^-$ to $\text{NO}_2^-$ with *Aspergillus* nitrate reductase. The skin contents of $\text{NO}_x$ were expressed as nanomoles per milligram of protein.

**Measurement of NOS Activity in Skin Samples**

NOS activity in combined tissue cytosolic and membrane fractions was measured as described previously (13, 16, 45). Briefly, frozen skin samples were crushed and homogenized at 4°C in a buffer (1 g/10 ml) containing 25 mmol/l Tris·HCl (pH 7.4), 1 mmol/l EGTA, 1 mmol/l EDTA, 1% (vol/vol) Nonidet P-40, 1 mmol/l phenylmethylsulfonyl fluoride, 2 mmol/l leupeptin, 1 mmol/l pepstatin, and 1 mmol/l aprotinin. The homogenates were centrifuged (14,000 g, 4°C) for 20 min, and the supernatants were used for measurement of protein content by the DC Lowry protein assay (Bio-Rad) and for measurement of NOS activity by the conversion of L-[14C]arginine to L-[14C]citrulline. Specifically, 120 µg of isolated protein were incubated for 60 min at 37°C in 100 µl of buffer containing 50 mmol/l Tris·HCl (pH 7.4), 10 µmol/l L-arginine, 1 mmol/l freshly made NADPH, 10 µmol/l tetrahydrobiopterin, 5 µmol/l FAD, 5 µmol/l FMN, and 0.1 µCi (~200,000 cpm) of L-[14C]arginine (Amersham Biosciences, Baie d’Urfé, QC, Canada). To determine Ca$^{2+}$-dependent endothelial and neural NOS (eNOS) activity, 2 mmol/l CaCl$_2$ and 100 mmol/l calmodulin were included in the assay. To determine Ca$^{2+}$-independent inducible NOS (iNOS) activity, the assay was conducted in the presence of 1 mmol/l EGTA without CaCl$_2$ and calmodulin. The assays were performed in the presence or absence of 1 mmol/l Nω-nitro-L-arginine (l-NNA) methyl ester, and the difference in counts per minute was used to calculate NOS activity. The reaction was stopped by addition of 1 ml of cold (4°C) stop buffer containing 50 mmol/l HEPES (pH 5.5), 5 mmol/l EDTA, and 5 mmol/l EGTA. The reaction mixture was passed over a 1-ml column containing the sodium form of Dowex AG 50 WX-8 resin (preequilibrated in stop buffer), washed with 3 ml of water, and collected into a 20-ml liquid scintillation vial. NOS activity was expressed as picomoles of citrulline per minute per milligram of protein.

**Measurement of eNOS Protein Expression in Skin Samples**

Expression of eNOS protein in skin samples was studied by Western blot analysis as described previously (13, 42). Frozen skin samples were crushed and homogenized at 4°C in a buffer (1 g/10 ml) containing 20 mmol/l Tris·HCl (pH 7.4), 150 mmol/l NaCl, 5 mmol/l EDTA, 1 mmol/l sodium orthovanadate, 1% (vol/vol) Nonidet P-40, 1 mmol/l phenylmethylsulfonyl fluoride, 2 µmol/l leupeptin, and 1 µmol/l aprotinin. The supernatants obtained after centrifugation (14,000 g, 4°C) for 20 min were used for assay of protein content using the DC Lowry protein assay. eNOS protein expression was assayed as follows. Briefly, after addition of Laemmli buffer to aliquots of supernatants (20 µg of total protein) to a final concentration of 50%, the samples were denatured by 2 min of boiling and resolved by electrophoresis on 6% SDS-polyacrylamide gels. The separated proteins were electrophoretically transferred at 4°C to polyvinylidene difluoride membranes (Immobilon-P, Millipore) at a constant voltage (35 V) overnight at 4°C. The blots were incubated for 60 min at room temperature in Tris-buffered saline with 0.1% Tween 20 (TBST) and 0.6% nonfat dry milk to block nonspecific antibody binding. After they were washed three times (5 min each) in TBST, the blots were incubated in a 1:2,500 dilution of monoclonal mouse anti-rat eNOS antibody (Transduction Laboratories, Lexington, KY) for 60 min. After they were washed three times (5 min each) in TBST, the blots were incubated again for 45 min at room temperature in a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (Bio-Rad). Then the blots were washed six times with TBST and developed by the enhanced chemiluminescence system (Amersham Life Sciences, Buckinghamshire, UK) on high-performance chemiluminescence film (Amersham Biosciences). The band densities were quantified using scanning laser densitometry (Fluorchem Software, Alpha Innotech, San Leandro, CA). The Western blots were probed with an anti-actin antibody for standardization of protein loading.

**Protocols**

**Protocol 1**: dose- and time-dependent effect of local subdermal Ad.VEGF-165 gene therapy on augmentation of skin flap viability. A 4 × 10 cm skin flap was mapped on the dorsum of each rat. PBS (0.5 ml) or 0.5 ml of PBS containing 2 × 10$^{10}$ pfu of Ad.Null or 2 × 10$^8$, 2 × 10$^9$, 2 × 10$^{10}$, and 2 × 10$^{11}$ pfu of Ad.VEGF-165 was injected subcutaneously into the distal half of the skin flap (n = 6). A 4 × 10 cm skin flap was constructed on the dorsum of each rat 7 days after the injection, and the area of viable and nonviable skin in the dorsal skin flap was assessed 7 days postoperatively.

In a separate study, PBS (0.5 ml) containing 2 × 10$^9$ pfu of Ad.Null or Ad.VEGF-165 was injected subcutaneously into the distal portion of the mapped dorsal skin flap in the rat 7 days or 7 and 14 days before surgery (n = 6), and skin flap viability was assessed 7 days after surgery.

**Protocol 2**: efficacy of preoperative local subdermal Ad.VEGF-165 and Ad.eNOS gene therapy in augmentation of rat dorsal skin flap viability. Rats were assigned to four groups (n = 6): local subdermal injection of 0.5 ml of PBS (group 1) or 0.5 ml of PBS containing 5 × 10$^8$ pfu of Ad.Null (group 2), Ad.VEGF-165 (group 3), or Ad.eNOS (group 4) 7 days before skin flap surgery. Skin flap ischemic necrosis was assessed 7 days postoperatively.

**Protocol 3**: effect of local subdermal VEGF-165 gene therapy on capillary density in rat dorsal skin flaps. A 4 × 10 cm skin flap was mapped on the dorsum of each rat. PBS (0.5 ml) or 0.5 ml of PBS containing 2 × 10$^9$ pfu of Ad.Null or Ad.VEGF-165 was injected subcutaneously into the distal half of the mapped skin flap, and skin flap surgery was performed 7 days after injection (n = 6). A skin sample (0.5 × 1.0 cm) was obtained 6.0 cm from the pedicle along the longitudinal midline of each dorsal skin flap 9 h after surgery. The skin samples were rinsed in isotonic saline and preserved in sodium phosphate buffer solution with 10% formalin (pH 7.4).

All slides were prepared by the Department of Histology at Toronto General Hospital. Permanent histological slide sections were prepared from paraffin-embedded skin samples. All sections were stained with hematoxylin and eosin, and the immunohistochemical technique was used for staining factor VIII-related antigen on the endothelial surface of skin vasculature as reported previously (17, 34). Stained sections were viewed by a single observer who was blinded to the treatment regimen. Under ×250 magnification (Olympus microscope, Leitz, Wetzlan, 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, capillaries were counted in five 0.46-mm$^2$ random fields. Capillary density was calculated as the number of capillaries per square millimeter.

**Protocol 4**: effect of local subdermal VEGF-165 gene therapy on blood flow in rat dorsal skin flaps. The control and treatment groups were the same as those described for protocol 3. Skin flap perfusion was assessed 9 h postoperatively. Specifically, blood flow in the dorsal skin flap was measured with 15-µm-diameter 59Co-labeled radioactive microspheres (DuPont NEN, Boston, MA) using the reference blood sampling technique described previously (7, 8, 29). Because these 15-µm-diameter microspheres would be trapped in the small arteries before entering the capillary bed, this technique most likely measured total skin capillary blood flow. The 4 × 10 cm dorsal skin flap was cut transversely into 10 segments of 1 × 4 cm each from the pedicle to the distal end of the skin flap. The radioactivity of each skin segment was measured on a gamma counter. A microcomputer was programmed to calculate the total skin flap blood flow and regional blood flow in each of the 10 segments of the skin flaps (7, 29).
Protocol 5: effect of the NOS inhibitor L-NNA and the cyclooxygenase inhibitor indomethacin in local subdermal Ad.VEGF-165 gene therapy for augmentation of skin flap viability. Rats were assigned to seven groups (n = 6): subdermal injection of 0.5 ml of PBS 7 days before surgery (group 1), subdermal injection of 0.5 ml of PBS containing 2 × 10\(^9\) pfu of Ad.Null 7 days before surgery (group 2), subdermal injection of 0.5 ml of PBS containing 2 × 10\(^9\) pfu of Ad.VEGF-165 at 7 days before surgery (group 3), subdermal injection of 0.5 ml of PBS containing 2 × 10\(^9\) pfu of Ad.VEGF-165 at 7 days before surgery and intramuscular injection of indomethacin (5 mg/kg) 45 min before surgery (group 4), subdermal injection of 0.5 ml of PBS containing 2 × 10\(^9\) pfu of Ad.VEGF-165 at 7 days before surgery and intramuscular injection of L-NNA (15 mg/kg) 45 min before surgery (group 5), subdermal injection of 0.5 ml of PBS containing 2 × 10\(^9\) pfu of Ad.Null 7 days before surgery and intramuscular injection of indomethacin (5 mg/kg) 45 min before surgery (group 6), and subdermal injection of 0.5 ml of PBS containing 2 × 10\(^9\) pfu of Ad.Null 7 days before surgery (group 7). Skin flap viability in all groups of rats was assessed 7 days postoperatively.

In a separate study, rats were assigned to five groups (n = 6): subdermal injection of 0.5 ml of PBS 7 days before surgery (group 1), subdermal injection of 0.5 ml of PBS containing 2 × 10\(^9\) pfu of Ad.Null 7 days before surgery (group 2), subdermal injection of 0.5 ml of PBS containing 2 × 10\(^9\) pfu of Ad.VEGF-165 at 7 days before surgery (group 3), subdermal injection of 0.5 ml of PBS containing 2 × 10\(^9\) pfu of Ad.VEGF-165 at 7 days before surgery and intramuscular injection of L-NNA (15 mg/kg) 45 min before surgery (group 4), and subdermal injection of 0.5 ml of PBS containing 2 × 10\(^9\) pfu of Ad.Null 7 days before surgery and intramuscular injection of L-NNA (15 mg/kg) 45 min before surgery (group 5). Skin flap blood flow was measured 9 h postoperatively using the radioactive microsphere technique.

Protocol 6: effect of preoperative subdermal Ad.VEGF-165 gene therapy on skin NO content and NOS activity and protein expression 9 h after skin flap surgery. A 4 × 10 cm skin flap was mapped on the dorsum of each rat. PBS (0.5 ml) or 0.5 ml of PBS containing 2 × 10\(^9\) pfu of Ad.Null or Ad.VEGF-165 was injected subdermally into the distal half of the mapped skin flap, and skin flap surgery was performed 7 days after injection (n = 6). At 9 h after skin flap surgery, 2 × 2.5 cm samples were obtained from the distal half of the dorsal skin flap 5.0–7.5 and 7.5–10.0 cm from the pedicle of the skin flap. These skin samples were used for assessment of NO content, eNOS and iNOS activity, and eNOS protein expression.

Biochemicals

Unless otherwise stated, all reagents and drugs were purchased from Sigma Chemical (Oakville, ON, Canada). Purified water (Milli-Q Water System, Millipore) was used to make all solutions and buffers.

Statistics

Values are means ± SE, unless otherwise stated. The specific statistical test used in each study and the number of observations in each group are stated in the legend of each figure.

RESULTS

Efficacy of Local Subdermal VEGF-165 Gene Therapy for Augmentation of Skin Flap Viability

In this 4 × 10 cm rat dorsal skin flap model, ischemic necrosis is known to occur in the distal portion of the skin flap. The extent of skin flap viability was similar between the control skin flaps receiving local subdermal injection of 0.5 ml of PBS and those receiving subdermal injection of 0.5 ml of PBS containing 2 × 10\(^9\) pfu of Ad.Null or Ad.VEGF-165 at 7 days before skin flap surgery. Areas of viable and nonviable skin were assessed 7 days postoperatively. Values are means ± SE (n = 6). Means without a common letter are significantly (P < 0.05) different (a > b; 1-way ANOVA followed by Newman-Keuls multiple comparison test).

PBS containing 2 × 10\(^9\) pfu of Ad.Null 7 days before skin flap surgery: 56 ± 3 and 54 ± 2%, respectively (Fig. 1). However, local subdermal injection of 0.5 ml of PBS containing Ad.VEGF-165 at 7 days before skin flap surgery increased skin flap viability in a dose-dependent manner, with a significant increase (67 ± 1%, P < 0.05) starting at 2 × 10\(^6\) pfu of Ad.VEGF-165 and increasing up to 2 × 10\(^9\) pfu of Ad.VEGF-165 (Fig. 1).

The time-dependent effect of Ad.VEGF-165 gene therapy in augmentation of skin flap viability was also investigated. There was no significant difference in viability between rat dorsal skin flaps injected subdermally with 2 × 10\(^7\) pfu of Ad.Null 7 days before skin flap surgery and those receiving the same injection 7 and 14 days before surgery: 54 ± 1 and 57 ± 2%, respectively (n = 6). Similarly, there was no significant difference in viability between rat dorsal skin flaps injected subdermally with 2 × 10\(^8\) pfu of Ad.VEGF-165 at 7 days before skin flap surgery and those receiving the same injection 7 and 14 days before surgery: 71 ± 1 and 75 ± 2% (n = 6). However, local subdermal injection of Ad.VEGF-165 at 7 days or 7 and 14 days before surgery consistently and significantly (P < 0.05) increased skin flap viability compared with Ad.Null-injected controls.

Comparison of Preoperative Local Subdermal Ad.VEGF-165 and Ad.eNOS Gene Therapy for Augmentation of Rat Dorsal Skin Flap Viability

There was no significant difference in viability between rat dorsal skin flaps injected subdermally with PBS and those injected with 0.5 ml of PBS containing 5 × 10\(^9\) pfu of Ad.Null 7 days before surgery: 53 ± 3 and 54 ± 2%, respectively (Fig. 2). Preoperative subdermal injection of 0.5 ml of PBS containing 5 × 10\(^9\) pfu of Ad.VEGF-165 or Ad.eNOS increased (P < 0.05) the rat dorsal skin flap viability to 67 ± 3 and 70 ± 3%, respectively. However, the augmentation of skin flap viability was similar for Ad.VEGF-165 and Ad.eNOS subdermal gene therapy (Fig. 2).
Effect of Local Subdermal VEGF-165 Gene Therapy on Capillary Density in Rat Dorsal Skin Flaps

Rat dorsal skin flaps were locally injected subdermally with 0.5 ml of PBS (control) or PBS containing 2 × 10^9 pfu of Ad.Null or Ad.VEGF-165 at 7 days before surgery. Sections from skin samples obtained from these three groups 9 h postoperatively were examined histologically for capillary density (Fig. 3). The capillary density was similar in samples from skin flaps injected subdermally with PBS and those treated with PBS containing 2 × 10^9 pfu of Ad.Null (Fig. 4). However, the capillary density in samples from skin flaps injected subdermally with Ad.VEGF-165 was significantly higher than that in samples from skin flaps treated with PBS (control) or PBS containing Ad.Null (Fig. 4).

Effect of Local Subdermal VEGF-165 Gene Therapy on Skin Blood Flow in Rat Dorsal Skin Flaps

Wet weight assessed 9 h postoperatively was similar in rat dorsal skin flaps injected subdermally with 0.5 ml of PBS (control) and those treated with 0.5 ml of PBS containing 2 × 10^9 pfu of Ad.Null or Ad.VEGF-165 at 7 days before surgery (Fig. 5). However, total skin blood flow 9 h postoperatively was significantly higher (P < 0.05) in skin flaps injected subdermally with PBS containing Ad.VEGF-165 than in those treated with PBS or PBS containing Ad.Null: 0.43 ± 0.06 vs. 0.21 ± 0.04 and 0.20 ± 0.02 ml·min⁻¹·flap⁻¹, respectively. At the time of the blood flow study, the mean arterial blood pressures of the rats injected subdermally with PBS or PBS
containing $2 \times 10^9$ pfu of Ad.Null or Ad.VEGF-165 were not significantly different: 108 ± 4, 110 ± 5, and 114 ± 4 mmHg, respectively. This observation may be interpreted to indicate that this dose of subdermal Ad.VEGF-165 gene therapy did not induce a circulating level of VEGF-165 high enough to cause hypotension. In addition, the weight of the skin flaps was similar in the control and treatment groups injected subdermally with Ad.Null or Ad.VEGF-165 (Fig. 5). This observation may also be interpreted to indicate that the dose of adenovirus used in this study did not cause significant edema.

The skin blood flow in each $1 \times 4$ cm segment of skin along the entire length of the skin flap was calculated and normalized to skin tissue weight (Fig. 6). At 1–9 cm from the pedicle, the mean skin blood flow was consistently higher in skin flaps injected subdermally with PBS containing $2 \times 10^9$ pfu of Ad.VEGF-165 than in those treated with PBS or PBS containing $2 \times 10^9$ pfu of Ad.Null 7 days postoperatively, and the difference was statistically significant up to 6 cm from the pedicle. This observation supports the observation that subdermal injection of PBS containing $2 \times 10^9$ pfu of Ad.VEGF-165 significantly ($P < 0.05$) augmented viability in the distal portion of the skin flap compared with skin flaps subdermally injected with PBS or PBS containing Ad.Null (Fig. 1).

Role of NO and Cyclooxygenase Products in Local Subdermal Ad.VEGF-165 Gene Therapy for Augmentation of Skin Flap Viability and Blood Flow

Viability was significantly ($P < 0.05$) increased in skin flaps injected subdermally with PBS containing $2 \times 10^9$ pfu of Ad.VEGF-165 compared with those injected subdermally with PBS or PBS containing $2 \times 10^9$ pfu of Ad.Null 7 days before skin flap surgery (Fig. 7). Augmentation of skin flap viability

Fig. 4. Capillary density in skin samples 9 h after skin flap surgery. PBS (0.5 ml) or 0.5 ml of PBS containing $2 \times 10^9$ pfu of Ad.Null or Ad.VEGF-165 was injected subdermally into the distal half of rat dorsal skin flaps 7 days before surgery. Samples ($0.5 \times 1.0$ cm) were taken along the longitudinal midline 6.0 cm from the pedicle of the skin flap 9 h after surgery. Values are means ± SE ($n = 6$). Means without a common letter are significantly ($P < 0.05$) different (a > b; 1-way ANOVA followed by Newman-Keuls multiple comparison test).

Fig. 5. Skin wet weight and blood flow in rat dorsal skin flaps 9 h postoperatively. PBS (0.5 ml, control) or 0.5 ml of PBS containing $2 \times 10^9$ pfu of Ad.Null or Ad.VEGF-165 was injected subdermally into the distal half of skin flaps 7 days before surgery. Blood flow and weight were measured 9 h postoperatively. Values are means ± SE ($n = 6$). Means without a common letter are significantly ($P < 0.05$) different (a > b; 1-way ANOVA followed by Newman-Keuls multiple comparison test).

Fig. 6. Regional skin blood flow in rat dorsal skin flaps 9 h postoperatively. PBS (0.5 ml, control) or 0.5 ml of PBS containing $2 \times 10^9$ pfu of Ad.Null or Ad.VEGF-165 was injected subdermally into the distal half of skin flaps 7 days before surgery. Blood flow was calculated for each $1 \times 4$ cm segment of skin from the pedicle to the distal end of the flap. Values are means ± SE ($n = 6$). Numbers in parentheses represent the number of flaps with detectable blood flow. *$P < 0.05$ vs. other treatments at the same distance from the pedicle (1-way ANOVA followed by Newman-Keuls multiple comparison test).

Fig. 7. Role of NO and cyclooxygenase products in subdermal Ad.VEGF-165 gene therapy for augmentation of skin flap viability in rat dorsal skin flaps. PBS (0.5 ml, control) or 0.5 ml of PBS containing $2 \times 10^9$ pfu of Ad.Null or Ad.VEGF-165 was injected subdermally into skin flaps 7 days before surgery. Nonselective NOS inhibitor $N^\text{G}$-nitro-L-arginine (L-NNA, 15 mg/kg) or cyclooxygenase inhibitor indomethacin (Indo, 5 mg/kg) was injected intramuscularly 45 min before flap surgery. Areas of viable and nonviable skin were assessed 7 days postoperatively. Values are means ± SE ($n = 6$). Means without a common letter are significantly ($P < 0.05$) different (a > b; 1-way ANOVA followed by Newman-Keuls multiple comparison test).
by local subdermal injection of PBS containing $2 \times 10^9$ pfu of Ad.VEGF-165 was not attenuated by a single intramuscular injection of the cyclooxygenase inhibitor indomethacin (5 mg/kg) 45 min before surgery. However, a single intramuscular injection of the NO synthase inhibitor tNNA (15 mg/kg) 45 min before skin flap surgery completely abolished the therapeutic effect of local subdermal Ad.VEGF-165 gene therapy in augmenting rat dorsal skin flap viability (Fig. 7). Intramuscular injection of indomethacin (5 mg/kg) or tNNA (15 mg/kg) 45 min before surgery had no significant effect on the viability of the control rat dorsal skin flaps injected subdermally with PBS containing $2 \times 10^9$ pfu of Ad.Null 7 days preoperatively (Fig. 7).

Again, blood flow was significantly ($P < 0.05$) higher in rat dorsal skin flaps injected subdermally with 0.5 ml of PBS containing $2 \times 10^9$ pfu of Ad.VEGF-165 than in those treated with 0.5 ml of PBS or 0.5 ml of PBS containing $2 \times 10^9$ pfu of Ad.Null 7 days preoperatively (Fig. 8). Augmentation of skin flap blood flow by subdermal Ad.VEGF-165 gene therapy was completely abolished by intramuscular injection of tNNA (15 mg/kg) 45 min before surgery. However, intramuscular injection of tNNA did not reduce blood flow in the control skin flaps injected subdermally with Ad.Null 7 days before surgery compared with the Ad.Null-injected control (Fig. 8).

**Effect of Local Subdermal VEGF-165 Gene Therapy on NOx Content in Rat Dorsal Skin Flaps**

Rat dorsal skin flaps were injected subdermally with 0.5 ml of PBS or 0.5 ml of PBS containing $2 \times 10^9$ pfu of Ad.Null or Ad.VEGF-165 at 7 days postoperatively, and skin samples were obtained 5.0–7.5 and 7.5–10.0 cm from the pedicle of the dorsal skin flaps 9 h postoperatively for study of skin contents of NOx. Ad.VEGF-165 gene therapy was associated with an increase in skin contents of NOx. Specifically, NOx content 5.0–7.5 cm from the pedicle was significantly higher in skin flaps injected subdermally with PBS containing $2 \times 10^9$ pfu of Ad.VEGF-165 than in those injected with PBS or PBS containing Ad.Null (Fig. 9). Similarly, NOx content 7.5–10.0 cm from the pedicle was significantly ($P < 0.05$) higher in skin flaps injected subdermally with PBS containing $2 \times 10^9$ pfu of Ad.VEGF-165 than in those injected subdermally with PBS or PBS containing $2 \times 10^9$ pfu of Ad.Null (Fig. 9).

NOx content decreased toward the distal end of the skin flaps in all groups. Specifically, the skin content of NOx within the control and treatment groups was significantly ($P < 0.05$) higher 5.0–7.5 cm than 7.5–10 cm from the pedicle of the skin flaps (Fig. 9).

**Effect of Local Subdermal VEGF-165 Gene Therapy on Skin Tissue NOS Activity and Protein Expression**

Subdermal injection of PBS containing $2 \times 10^9$ pfu of Ad.VEGF-165 at 7 days postoperatively significantly ($P < 0.05$) increased cNOS activity 5.0–7.5 cm from the pedicle of the rat dorsal skin flap 9 h after skin flap surgery compared with those injected subdermally with PBS or PBS containing $2 \times 10^9$ pfu of Ad.Null (Fig. 10). Similarly, skin cNOS activity 7.5–10.0 cm from the pedicle was significantly ($P < 0.05$) higher in skin flaps injected subdermally with Ad.VEGF-165 than in those injected subdermally with PBS or PBS containing Ad.Null (Fig. 10). Within the control and treatment groups, there was no significant difference in cNOS activity between skin samples obtained 5.0–7.5 cm and those obtained 7.5–10.0 cm from the pedicle of the skin flap.

Skin iNOS activity was also assessed 5.0–7.5 and 7.5–10.0 cm from the pedicle of the rat dorsal skin flaps 9 h postoperatively. There was no significant difference in skin iNOS activity among the three groups injected subdermally with PBS or PBS containing $2 \times 10^9$ pfu of Ad.Null or Ad.VEGF-165 at 7 days before skin flap surgery (Fig. 11).

Western blot analysis was used to assess skin protein levels of eNOS 5.0–7.5 and 7.5–10.0 cm from the pedicle of rat dorsal skin flaps 9 h after skin flap surgery. At both locations, the protein level of eNOS was significantly ($P < 0.05$) higher in skin flaps injected subdermally with PBS containing $2 \times 10^9$ pfu of Ad.VEGF-165 than in those injected subdermally with PBS or PBS containing $2 \times 10^9$ pfu of Ad.Null 7 days preoperatively (Fig. 12). There was no significant difference in eNOS protein levels 9 h postoperatively between skin flaps injected subdermally with PBS and those injected with PBS containing $2 \times 10^9$ pfu of Ad.Null 7 days before surgery.

**DISCUSSION**

**Important Findings From the Present Studies**

Three new findings from the present studies of local subdermal VEGF-165 gene therapy for augmentation of skin viability in skin flap surgery are as follows: 1) Local subdermal injection of Ad.VEGF-165 at 7 days preoperatively augmented rat dorsal skin flap viability in a dose-dependent manner. This
gene therapy was associated with an increase in eNOS protein expression, cNOS activity, and NOx content and capillary density and blood flow in the skin flap. 2) The efficacy of preoperative subdermal gene therapy for augmentation of rat dorsal skin flap viability was similar for Ad.VEGF-165 and Ad.eNOS at $5 \times 10^8$ pfu. 3) A single intramuscular injection of the NOS inhibitor L-NNA 45 min preoperatively completely abolished the therapeutic effect of preoperative local subdermal VEGF-165 gene therapy in augmenting rat dorsal skin flap blood flow and viability. Taken together, these observations support our hypothesis that preoperative subdermal Ad.VEGF-165 or Ad.eNOS gene therapy is effective in augmenting skin viability in skin flap surgery of the rat and that the mechanism of Ad.VEGF-165 gene therapy involves an increase in synthesis/release of NO and blood flow in the skin flap.

**Efficacy of Local Subdermal Ad.VEGF-165 and eNOS Gene Therapy in Augmentation of Rat Dorsal Skin Flap Viability**

We studied the dose- and time-dependent effect of local subdermal Ad.VEGF-165 gene therapy in augmenting rat dorsal skin flap viability. We confirmed the previous observation by other investigators that local subdermal injection of Ad.VEGF-165 was effective in augmenting rat dorsal skin flap viability (9, 46). In addition, we observed that the effective dose of subdermal Ad.VEGF-165 gene therapy was $2 \times 10^7$ pfu (Fig. 1) and that there was no significant difference in treatment effect on skin flap viability between Ad.VEGF-165 given in a single injection 7 days preoperatively and Ad.VEGF-165 administered 7 and 14 days preoperatively. Finally, we demonstrated for the first time that subdermal Ad.eNOS and Ad.VEGF-165 gene therapy 7 days preoperatively augmented skin flap viability to a similar extent (Fig. 2).

Fig. 9. Total NO (NOx) content in rat dorsal skin flaps 9 h postoperatively. PBS (0.5 ml, control) or 0.5 ml of PBS containing $2 \times 10^7$ pfu of Ad.Null or Ad.VEGF-165 was injected subdermally into the distal half of skin flaps 7 days before surgery. Skin samples were obtained 5.0–7.5 and 7.5–10.0 cm from the pedicle of the skin flap. Values are means ± SE (n = 6). Within the same distance, means without a common letter are significantly (P < 0.05) different (a > b; 2-way ANOVA followed by 1-way ANOVA and Newman-Keuls multiple comparison test).

Fig. 10. $Ca^{2+}$-dependent NOS (cNOS) activity in rat dorsal skin flaps 9 h postoperatively. PBS (0.5 ml, control) or 0.5 ml of PBS containing $2 \times 10^7$ pfu of Ad.Null or Ad.VEGF-165 was injected subdermally into the distal half of skin flaps 7 days before surgery. Skin samples (2.0 $\times$ 2.5 cm) were obtained 5.0–7.5 and 7.5–10.0 cm from the pedicle of the skin flap. Values are means ± SE (n = 6). Within the same distance, means without a common letter are significantly (P < 0.05) different (a > b; 2-way ANOVA followed by 1-way ANOVA and Newman-Keuls multiple comparison test).

Fig. 11. $Ca^{2+}$-independent (inducible) NOS (iNOS) activity in rat dorsal skin flaps 9 h postoperatively. PBS (0.5 ml, control) or 0.5 ml of PBS containing $2 \times 10^7$ pfu of Ad.Null or Ad.VEGF-165 was injected subdermally into the distal portion of skin flaps 7 days before surgery. Skin samples (2.0 $\times$ 2.5 cm) were obtained 5.0–7.5 and 7.5–10.0 cm from the pedicle of the skin flap. Values are means ± SE (n = 6). Means within each distance are not significantly different (2-way ANOVA followed by 1-way ANOVA).

Fig. 12. Endothelial NOS (eNOS) protein expression in rat dorsal skin flaps 9 h postoperatively. PBS (0.5 ml, control) or 0.5 ml of PBS containing $2 \times 10^7$ pfu of Ad.Null or Ad.VEGF-165 was injected subdermally into the distal half of skin flaps 7 days before surgery. Skin samples (2.0 $\times$ 2.5 cm) were obtained 5.0–7.5 and 7.5–10.0 cm from the pedicle of the skin flap. Values are means ± SE (n = 6). Within each distance, means without a common letter are significantly (P < 0.05) different (a > b; 2-way ANOVA followed by 1-way ANOVA and Newman-Keuls multiple comparison test).
Specifically, we observed that the NOS inhibitor L-NNA vasodilator effect was predominantly mediated by NO (2). Flaps that VEGF-165 was a potent vasodilator and that its density and blood flow 9 h postoperatively (Figs. 4 and 5). Ad.VEGF-165 into the distal half of the rat dorsal skin flap 7 days preoperatively was completely abolished by an intramuscular injection of L-NNA 45 min preoperatively (Fig. 8). Taken together, these observations led us to speculate that the vasodilator effect and the increase in vascular NO, resulting in an increase in capillary density and total blood flow and viability in rat dorsal skin flaps.

Mechanism of Local Subdermal Gene Therapy in Augmentation of Viability in Rat Dorsal Skin Flaps

We have demonstrated for the first time that local subdermal Ad.VEGF-165 gene therapy increased synthesis and release of NO in rat dorsal skin flaps. Specifically, NOx content was higher in the distal half (5.0–10.0 cm from the pedicle) of rat dorsal skin flaps injected with 0.5 ml of PBS containing 2 × 10^9 pfu of Ad.VEGF-165 at 7 days preoperatively than in skin flaps injected subdermally with 0.5 ml of PBS (control) or 0.5 ml of PBS containing Ad.Null (Fig. 9). NO is known to mediate VEGF-165-induced angiogenesis (25, 31, 48, 50). Other investigators also observed that preoperative local subdermal or subcutaneous Ad.VEGF-165 gene therapy induced angiogenesis and augmented viability in rat skin flaps, and they also speculated that improved skin flap blood flow as a result of angiogenesis augmented skin flap viability (9, 46). In the present study, we observed that local subdermal injection of Ad.VEGF-165 into the distal half of the rat dorsal skin flap 7 days preoperatively also induced an increase in skin capillary density and blood flow 9 h postoperatively (Figs. 4 and 5). However, we previously observed in isolated perfused pig skin flaps that VEGF-165 was a potent vasodilator and that its vasodilator effect was predominantly mediated by NO (2). Specifically, we observed that the NOS inhibitor L-NNA blocked the vasodilator effect and the increase in vascular content of NOx induced by intra-arteral infusion of VEGF-165 into isolated perfused pig skin flaps (2). In the present in vivo studies, we also observed that augmentation of rat dorsal skin flap viability by local subdermal Ad.VEGF-165 gene therapy 7 days preoperatively was completely abolished by an intramuscular injection of the NOS inhibitor L-NNA (15 mg/kg), but not by the cycloxygenase inhibitor indomethacin (5 mg/kg), 45 min before skin flap surgery (Fig. 7). The dose of indomethacin used in this study was effective in inhibiting cyclooxgenase in the rat (27). In addition, we observed that an increase in rat dorsal skin flap blood flow by local subdermal Ad.VEGF-165 gene therapy 7 days preoperatively was also completely abolished by an intramuscular injection of L-NNA 45 min preoperatively (Fig. 8). Taken together, these observations led us to speculate that the vasodilator, but not the angiogenic, effect of NO plays a predominant role in local subdermal Ad.VEGF-165 gene therapy for augmenting skin flap viability in the present studies. Our argument would have been strengthened if we had also studied the acute effect of L-NNA on the Ad.VEGF-165-induced increase in skin NOx content (Fig. 9). There is evidence in the literature to support our speculation. It was reported that Ad.VEGF-165 gene therapy 0.5, 3, 7, or 14 days preoperatively augmented rat skin flap viability to a similar extent (11). Skin blood flow was not measured in this study. However, it is well known that a period of 24–72 h is required for VEGF-165 to establish angiogenesis (18, 31, 32), and the critical time for skin ischemic tolerance in rat, rabbit, and pig skin flaps is 6–13 h (1, 15, 22, 24, 38, 44). Therefore, vasodilation may have played an important role in local subdermal Ad.VEGF-165 gene therapy in augmenting skin flap viability in previous studies. Furthermore, it was reported that the neovessels are immature, leaky, and fragile in VEGF-165-induced angiogenesis (3, 5). These observations also support our speculation that vasodilation, but not angiogenesis, may have played an important role in the Ad.VEGF-165-induced increase in skin blood flow and viability in rat dorsal skin flaps.

Mechanism for Augmentation of NO Production Induced by Local Subdermal Ad.VEGF-165 Gene Therapy

Other investigators reported upregulation of eNOS gene expression 6–9 h after exposure of cultured human endothelial cells and 6–24 h after exposure of rat aortic segments to VEGF-165 (4, 13). More recently, we observed upregulation of skin eNOS protein expression 9–24 h after subcutaneous injection of VEGF-165 into rat dorsal skin flaps (16). In the present study, skin eNOS protein expression was upregulated 7 days after subdermal injection of Ad.VEGF-165 into rat dorsal skin flaps (Fig. 12). This increase in eNOS protein expression most likely contributed to the increase in synthesis/release of NO in subdermal Ad.VEGF-165 gene therapy.

We recently demonstrated in isolated perfused pig skin flaps that the vasorelaxation effect of VEGF-165 is mediated by VEGF receptor-2 (flk-1/KDR), and the postreceptor signal transduction pathway involves the activation of phospholipase C and protein kinase C, an increase in inositol 1,4,5-trisphosphate activity, the release of intracellular Ca2++ stores, and the synthesis/release of NO (2). Ca2++ is also known to activate NOS for NO synthesis (12). In the present study, eNOS activity in the distal half of the rat dorsal skin flap assessed 9 h postoperatively was also higher in skin flaps injected subcutaneously with PBS containing Ad.VEGF-165 at 7 days preoperatively than those injected subdermally with PBS or PBS containing Ad.Null (Fig. 10). These observations led us to speculate that the increase in intracellular Ca2++ induced by VEGF-165 also activated eNOS for synthesis of the vasodilating and angiogenic NO.

Other investigators reported that subcutaneous injection of VEGF-165 into rat dorsal skin flaps did not affect skin iNOS gene expression (30). We previously demonstrated that subdermal VEGF-165 protein therapy in rat dorsal skin flaps did not affect skin iNOS activity. In the present study, subdermal Ad.VEGF-165 gene therapy also did not affect iNOS activity in the rat dorsal skin flap (Fig. 11). Therefore, it is most likely that iNOS was not involved in the increase in NO production induced by local subdermal gene therapy in rat dorsal skin flaps (Fig. 9).

In summary, we have studied the efficacy and mechanism of local subdermal Ad.VEGF-165 gene therapy for augmenting skin viability in skin flap surgery in the rat. We demonstrated for the first time that 1) local subdermal injection of Ad.VEGF-165 at 7 days preoperatively augmented rat dorsal skin flap viability in a dose-dependent manner, 2) the efficacy of local subdermal Ad.VEGF-165 and Ad.eNOS gene therapy in augmenting rat dorsal skin flap viability was similar at 5 × 10^9 pfu, and 3) subdermal Ad.VEGF-165 gene therapy involved upregulation of skin eNOS protein expression, eNOS activity, and synthesis/release of the vasodilating and angiogenic factor NO, resulting in an increase in capillary density and total blood flow and distal perfusion in the skin flap. In addition, there was evidence to indicate that the vasodilating effect of NO predominantly mediates local subdermal Ad.VEGF-165 gene therapy in augmenting skin flap blood flow and viability.
Perspectives

It is expected that the incidence of skin flap surgery will continue to increase because of a growing population of elderly citizens, who frequently have problems with wound healing, pressure sores, threatened ischemic limbs, and ulceration requiring skin flaps for wound reconstruction (3). The most common complication of skin flap surgery is ischemic necrosis in the distal portion of the skin flap. Failure of the skin flap is time consuming and costly, because it requires additional surgery and prolonged hospitalization. Local VEGF-165 or eNOS protein or gene therapy for augmentation of skin blood flow in the distal portion of the skin flap may resolve this surgical problem.

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