Expression of Endothelin 1 in Rat Random-Pattern Skin Flaps Treated With Topical Nifedipine

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Objective: To evaluate the relative tissue concentrations of the endogenous vasoactive peptide endothelin 1 (ET-1) in random-pattern skin flaps (RPSF) treated with either topical anti-ischemic drug therapy (nifedipine) or placebo.

Design: Prospective, randomized, placebo-controlled therapeutic trial.

Patients: Adult male Sprague-Dawley rats.

Intervention: Experimental subjects underwent caudally based RPSFs using the modified McFarlane technique. Subjects received either topical anti-ischemic drug therapy (nifedipine; n=6) or inert carrier ointment (placebo; n=6). Treatment was initiated immediately following flap closure and continued every 6 hours for 5 days. At the end of the treatment period, the animals were killed and the concentration of ET-1 was determined using enzyme-linked immunosorbent assay. Representative tissues from nifedipine- and placebo-treated skin flaps were also analyzed for ET-1 using immunohistochemical stains.

Results: The ET-1 levels in the distal (necrotic) flap segments were increased by 4.53 pg/mL over baseline (nonnecrotic) flaps in the placebo-treated animals and decreased by 4.70 pg/mL below baseline in the nifedipine-treated group (P = .03).

Conclusions: The correlation between tissue levels of ET-1 and the severity of tissue necrosis suggests that ET-1 may play a pivotal role in ischemic injury of RPSFs. Moreover, treatment with topical nifedipine may antagonize the vasoconstrictive effects of ET-1. Although immunohistochemical analysis revealed ET-1 staining within the flap microvasculature, no quantitative differences were detected between the nifedipine- and placebo-treated flaps. Further studies are needed to define the role of ET-1 in RPSF necrosis.

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Random-pattern skin flaps (RPSFs) are widely used in the repair of soft tissue defects resulting from tumor extirpation or trauma. Unfortunately, ischemic tissue necrosis remains a leading cause of flap failure. Technical factors believed to contribute to RPSF ischemic necrosis include flap design, wound tension, and intrinsic tissue vascularity.

Recent evidence suggests that the endogenous vasoactive peptide endothelin 1 (ET-1) may play an important role in the ischemic necrosis of RPSFs. A 21-amino acid peptide, ET-1 was first isolated and sequenced by Yanagisawa and colleagues in 1988 and is the most potent endogenous vasoconstrictive peptide found to date. Endothelin 1 has also been shown to be a potent inducer of terminal arteriolar contraction. Tane and coworkers found that intraperitoneal ET-1 injected into rats with dorsal RPSFs led to a significantly decreased length of flap survival. They concluded that ET-1 was an important regulator of the microcirculation of RPSFs. Inoue et al studied levels of ET-1 in rat RPSFs in the first 6 hours postoperatively and found ET-1 levels to be significantly increased in the distal flap segment compared with the proximal flap segment. These observations suggest that increased ET-1 levels may play an important role in RPSF ischemia.

Various pharmacologic agents have been evaluated for their ability to prevent or reduce skin flap ischemia, including sympatholytics, vasodilators, calcium channel blockers, hemorheologics, prostaglandin inhibitors, anticoagulants, glucocorticoids, and free radical scavengers. Despite the wide range of drugs examined, few of these pharmacologic agents have demonstrated unequivocal efficacy. Adverse effects, high cost, or the need for preoperative administration or direct vascular infusion have limited their clinical application to postopera-
tive skin flaps. As a consequence, the ideal agent for the salvage of failing RPSFs remains elusive.

While the exact mechanism of RPSF ischemia is poorly understood, surgically induced adrenergic vasoconstriction is believed to play a major role in ischemic injury. \(^1^,\text{10}^,\text{14}^,\text{16}^\) Vasactive drugs such as calcium channel blockers diminish sympathetic tone or promote arteriolar smooth muscle relaxation and have been shown to improve skin flap perfusion and survival. \(^1^,\text{3}^,\text{17}^\) Nifedipine, a clinically available calcium channel blocker, is believed to block adrenergically mediated vasoconstriction by inhibiting calcium ion flux into vascular smooth muscle cells. \(^1^,\text{17}^,\text{18}^\) At least 1 study has demonstrated that RPSFs treated with topical nifedipine show a statistically significant reduction in distal flap ischemic necrosis. \(^1^\) In addition, 3 additional animal studies using oral nifedipine have demonstrated improvement in RPSF survival with postoperative oral drug administration. \(^1^,\text{17}^,\text{19}^\) To further elucidate the role of ET-1 in RPSF ischemia, this study examines the concentration of ET-1 in rat RPSF tissue following topical treatment with the vasodilator nifedipine.

**METHODS**

These experiments were conducted in full accordance with the protocols established by the Institutional Animal Care and Use Committee at the University of Miami, Miami, Fla. Adult male Sprague-Dawley rats (250-275 mg) were used as experimental subjects. Isoflurane inhalational anesthesia was used to approximate routine surgical conditions. Anesthetic induction was achieved with 5% isoflurane, and subjects were maintained intraoperatively with 1.5% isoflurane administered through a snout cone by spontaneous ventilation. Adequate depth of anesthesia was confirmed using the pinch flexion/withdrawal test.

Following induction, animals were shaved, prepped, and immobilized in the prone position. Caudally based RPSFs, each measuring \(3 \times 10 \text{ cm}\), were raised along the dorsal midline by sharp dissection. Flaps were designed according to the Khouri modification of the original McFarlane technique, and care was taken to elevate the panniculus carnosus with the overlying skin. \(^2^,\text{17}^,\text{18}^\) Hemostasis was obtained with electrocautery, and the flaps were then sutured back to their native position using 4-0 running silk sutures (Ethicon Inc, Somerville, NJ). Immediately following flap closure, each flap pedicle was coated with either 3 mL of glycerin-based carrier ointment (placebo) or 3 mL of carrier ointment containing 5 mg of nifedipine (Procardia; Pfizer Inc, New York, NY). Care was taken to distribute ointment evenly along the entire flap, and dosing was repeated every 6 hours throughout the study period.

Postoperatively, restrictive collars were placed to prevent unwanted ingestion of ointment during self-grooming, and the animals were returned to solitary housing following emergence from anesthesia. Subjects were allowed water and rat chow ad libitum throughout the study period, and acetaminophen with codeine was added to the drinking water for 5 days postoperatively.

At the conclusion of the study period, all subjects were killed by rapid carbon dioxide asphyxia. Flaps were then harvested and processed for either enzyme-linked immunosorbent assay (ELISA) or immunohistochemical analysis.

**PART 1—TIME COURSE OF ET-1 EXPRESSION**

The RPSF procedure was performed on 15 animals. Three animals were then killed on each of postoperative days 0, 1, 3, 5, and 7. Each flap was divided into proximal and distal flap segments, which were then processed for ELISA analysis.

**PART 2—DETERMINATION OF ET-1 TISSUE LEVELS AFTER ANTI-ISCHEMIC DRUG THERAPY**

The RPSF procedure was performed on 2 groups of 6 experimental animals each. For 5 days, the RPSFs were treated with either 3 mL of topical carrier ointment (glycerine) or 3 mL of topical carrier ointment containing 5 mg of nifedipine (5 mg/3 mL). Dosing was repeated every 6 hours for the 5-day study period. After 5 days, the animals were killed, and the RPSFs were harvested for ELISA and immunohistochemical analysis. The flaps were then divided lengthwise, permitting one half to be processed for immunohistochemical analysis and the contralateral side to be processed for ELISA. The bisected segments were then subdivided into 4 equal flap segments along the length of the flap. The most distal flap segment was discarded, as it was composed mostly of nonviable necrotic tissue. The remaining 3 flap segments (proximal, middle, and distal) were processed and analyzed by ELISA for ET-1 levels, which were recorded. The proximal (nonnecrotic) flap segment served as the baseline ET-1 level for the study. The change in ET-1 from the proximal to middle flap segment and the proximal to distal flap segment were also recorded, along with the SEM.

**IMMUNOHISTOCHEMICAL ANALYSIS**

Tissue samples were paraffin embedded, sectioned, and mounted onto slides. The tissues were then incubated overnight in a commercially available ET-1 antibody at room temperature using a 1:1000 antibody dilution (Peninsula Laboratories, San Carlos, Calif). After washing with 0.01M phosphate-buffered saline, the tissues were incubated for 1 hour with a donkey-antirabbit secondary antibody conjugated to biotin at a 1:800 dilution, (Jackson ImmunoResearch Laboratories Inc, West Grove, Pa) and then washed and incubated in avidin-biotin complex reagent for 1 hour (Vector Laboratories, Burlingame, Calif). Finally, the tissues were incubated in diamobenzidine to yield a tannish reaction product, cleared and mounted in glycerol for light microscopy viewing, and photographed using a digital Pixera camera (Los Gatos, Calif).

A histopathologist, blinded to the study, assessed the staining of ET-1 in the nifedipine- and placebo-treated RPSFs in a quantitative manner through sequential visual grading of consecutive high-powered fields. Amount and density of staining in the region of the subdermal plexus were recorded. Areas of staining outside of the subdermal plexus were also recorded.

**RESULTS**

The ELISA analysis was performed using a commercially available kit (R&D Systems, Minneapolis, Minn) on tissues isolated and prepared using established protocols. \(^4^\) Results were analyzed by a standard paired \(t\) test using commercially available software (Statview, version 4.5; SAS Institute Inc, Cary, NC).

**PART 1—TIME COURSE OF ET-1 EXPRESSION**

The levels of ET-1 in the proximal and distal ends of the skin flaps were determined over a 7-day period. The mean ET-1 levels for the proximal and distal flap segments are shown in Figure 1. Beginning on postoperative day 3, ET-1 levels were higher in the distal flap segment than in the proximal portion of the flap. The highest concentration of ET-1 in the distal flap segment was observed on postoperative day 5.
Changes in ET-1 levels along the length of the flap were statistically significant in the distal flap segment of animals treated with nifedipine. In contrast, for the nifedipine-treated group, ET-1 levels in the distal flap increased by 4.70±3.18 pg/mL (n=6). The observed decrease in ET-1 levels in the proximal flap at 24 hours, ET-1 levels in the distal (necrotic) flap segment were found to correspond to the clinical onset of ischemic necrosis, maximum concentration of ET-1 in the distal segment occurring 5 days following flap elevation. Because the maximum concentration of ET-1 in the distal segment corresponded to the clinical onset of ischemic necrosis, endogenous vasoconstriction produced by ET-1 would seem to have contributed to RPSF failure.

**IMMUNOHISTOCHEMICAL ANALYSIS**

Placebo- and nifedipine-treated flaps failed to demonstrate a significant difference in the amount of immunoreactive staining for ET-1 in the subdermal vascular plexus after blinded scoring of the tissue specimens. Representative sections are shown in Figure 3.

**COMMENT**

Random-pattern skin flaps remain the cornerstone for reconstruction of cutaneous defects. Careful flap design and meticulous surgical technique often result in successful implementation of RPSFs in cutaneous reconstruction. However, excessive wound closing tension, poor flap design, or impaired intrinsic tissue vascularity may compromise tissue perfusion and result in a wide spectrum of negative sequelae ranging from superficial sloughing to complete flap necrosis and loss.

Endothelin 1, an endogenous vasoactive peptide, is believed to play an important role in modulating RPSF vascular perfusion. Two studies have examined the role of ET-1 in the survival of RPSFs in animal models. The initial study by Tane et al determined that intraperitoneal injection of ET-1 reduced survival length of a rat skin flap. When ET-1 was given in combination with an endothelin A receptor antagonist, the effect of ET-1 on survival length was reversed. The antagonist alone had no effect on survival length, suggesting that endogenous levels of ET-1 did not affect flap survival. However, when regional differences in ET-1 levels were determined using ELISA, the highest levels of ET-1 were found in the flap base. In a similar study, Inoue et al also demonstrated that the distribution of ET-1 was localized to the base of the flap soon after flap elevation. In animals treated with an endothelin A receptor antagonist, flap survival was improved from 39 to 52 mm². These findings suggest that antagonism of ET-1 receptor binding improved flap survival because ET-1 was likely involved in flap necrosis by promoting vasoconstriction. However, both of these studies have focused on the expression of ET-1 in the first 24 hours following flap surgery.

In contrast to the above studies, the present investigation found that endogenous ET-1 can be detected within RPSFs up to 7 days postoperatively. Moreover, while we also observed an initial increase in ET-1 concentration in the proximal flap at 24 hours, ET-1 levels gradually diminished in the flap base over the 7-day study period (Figure 1). Perhaps more importantly, ET-1 levels in the distal (necrotic) flap segment were found to increase over time with a maximum expression of ET-1 occurring 5 days following flap elevation. Because the maximum concentration of ET-1 in the distal segment corresponded to the clinical onset of ischemic necrosis, endogenous vasoconstriction produced by ET-1 would seem to have contributed to RPSF failure.

According to a previously established model for RPSF ischemia, untreated RPSFs exhibit approximately 44% necrosis of the distal flap segment by the seventh postoperative day. However, when these flaps are treated postoperatively with topical application of the vasodilator nifedipine, RPSF survival is increased by approximately 18%. Because similar studies have also shown that nifedipine inhibits cutaneous vasoconstriction, we chose in the present study to measure the effect of nifedipine on the concentration of ET-1 using this established RPSF model. Since topical nifedipine is thought to improve skin flap survival by antagonizing microcirculatory vasocon-
striction, we postulated that nifedipine treatment would result in a corresponding reduction in tissue levels of ET-1. Not surprisingly, we observed a corresponding decrease in ET-1 levels within the distal (necrotic) flap segment over baseline concentrations following treatment with topical nifedipine (Figure 2). Moreover, the decrease in ET-1 levels observed in the nifedipine-treated flaps was statistically significant compared with those of the placebo-treated flaps. In a related study, application of nifedipine has also been shown to fully relax internal mammary arteries precontracted with ET-1. This study further demonstrated a dose-dependent reduction of ET-1 in cultured endothelial cells that were pretreated with the calcium channel blocker nisoldipine. These studies support the notion

Figure 3. Endothelin 1 (ET-1) expression in the distal and proximal skin flaps. Distal skin flap segments (A, C, and E taken on experimental days 5, 6, and 7, respectively) show ET-1 staining in the rat subdermal vasculature; proximal skin flap segments (B, D, and F taken on the same animals also on days 5, 6, and 7, respectively) show less ET-1 staining in the microvasculature. Qualitatively, there is more ET-1 staining in the distal vessels than the proximal vessels, but this qualitative difference was not apparent in all of the tissue samples.
that calcium channel blockers may promote decreased ET-1 synthesis.

Based on our findings, ET-1 appears to have an important role in RPSF ischemia. Moreover, the salvage effect observed with topical nifedipine seems to be associated with a corresponding reduction in ET-1 tissue levels within the ischemic flap segment. Although immunohistochemical analysis failed to reveal a visible difference in ET-1 concentrations between treated and untreated subjects, this is most likely due to the differing sensitivities of ELISA and immunohistochemical analysis. The ELISA quantification showed differences of less than 5 pg/mL, which is well below the sensitivity of routine immunohistochemical analysis. While an association between the application of topical nifedipine and reductions in ET-1 tissue levels has been demonstrated in the present study, further studies are needed to elucidate the precise interaction between nifedipine and ET-1. Additional studies to assess the possible efficacy of nifedipine flap salvage in humans are also indicated.

In conclusion, despite continued improvement in the understanding of skin flap vascular physiology, failure of these flaps remains a clinical problem. To prevent the morbidity associated with loss of RPSFs, therapeutic modalities have focused on the improvement of flap perfusion. Our studies support the notion that the endogenous vasoactive peptide ET-1 may be one of the mediators involved in skin flap ischemia and that topical nifedipine may serve to decrease tissue levels of this potent vasoconstrictor.

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Reprints are not available from the authors.

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