Platelet-Derived Growth Factor B, but Not Fibroblast Growth Factor 2, Plasmid DNA Improves Survival of Ischemic Myocutaneous Flaps

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Hypothesis: Tissue flaps are commonly used for surgical reconstruction, especially to cover difficult wounds and in breast reconstruction following mastectomy. Complications due to inadequate flap perfusion are a source of morbidity and, in the lower extremity, can result in amputation.

Setting: Laboratory.

Interventions: We evaluated the ability of platelet-derived growth factor (PDGF) B and fibroblast growth factor 2 plasmid DNA, formulated in a type I collagen matrix, to promote tissue survival in a rat transverse rectus abdominis muscle flap model based on the inferior deep epigastric vascular supply. In the absence of any therapeutic agent, only about 24% of flap tissue survives in this model. The DNA/matrix formulations were delivered subcutaneously into the skin paddles 7 days before flap elevation, and tissues were harvested 7 days later.

Results: Our studies reveal dramatic increases in overall vascularity after treatment with PDGF-B and fibroblast growth factor 2 plasmid DNA; however, only PDGF-B increased flap survival (130% increase at 228 µg/cm² of plasmid DNA vs controls; *P* < 0.01). Transdermal spectral imaging demonstrated an increase in patent vessels supporting blood flow in flaps treated with PDGF-B plasmid DNA vs the fibroblast growth factor 2 transgene.

Conclusion: Matrix-enabled gene therapy may provide an effective nonsurgical approach for promoting flap survival and is well suited for surgical applications in which transient therapeutic transgene expression is desired.

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THERAPEUTIC ANGIOGENESIS refers to the use of proangiogenic factors to reverse the detrimental effects of ischemia on tissue survival and function either through maintenance of existing vasculature or by stimulating the formation of a neovasculature.1-3 Support of vascular formation and function has progressed since the identification of such factors and the supportive molecular mechanisms of action, including variants of the fibroblast growth factor (FGF), vascular endothelial cell growth factor, angiopoietin, and platelet-derived growth factor (PDGF) families.4-6 Recombinant protein and transgene forms of such factors have been delivered to ischemic tissues either through direct tissue injection or via local circulation with various degrees of success in the laboratory and in clinical trials for the treatment of various conditions, including ischemic myocardium, limb ischemia, and peripheral artery disease.5-19 These same factors also have potential use in the treatment of tissue flaps, which are transferable cutaneous tissue segments that can include surrounding musculature, adipose tissue, or fascial tissue, depending on specific surgical needs and limitations.15-17 Flaps are used in the surgical repair of tissue defects caused by disease or trauma, including postmastectomy breast reconstruction and lower limb ulcer treatment. The required perfusion of the flap tissue is achieved by maintenance of at least one pair of original feed vessels (ie, a pedicled flap) or through the microsurgical anastomosis of the flap vasculature to a distant supply artery (ie, a free flap). Survival of any flap—and success of the surgical procedure—requires an adequate vasculature supply to provide arterial feed and venous drainage of the involved tissue.

Despite advances in microsurgical techniques and in knowledge of local vascular networks, estimates indicate that approximately 20% to 25% of flaps develop some area of necrosis due to compro-
mixed circulation, particularly in high-risk patients (smokers or obese patients). Therefore, circulatory support is a primary limiting factor of flap size and has focused efforts to augment the vascular supply and network of a flap by surgical, pharmacological, and gene therapeutic approaches. Surgical approaches include creating microsurgical anastomoses of additional feed vessels or performing a “delay” through the division of a major feed artery or multiple secondary feed vessels before flap creation. However, such procedures may cause additional surgery for the patient and surgeon.

Although multiple pharmacological approaches have been used to enhance flap survival experimentally, none have found widespread clinical acceptance or utility, including antioxidants and vasodilators. Gene transfer approaches have, therefore, received increased attention as primary therapeutic treatments or adjuvants to surgical approaches. Systemic antioxidants and local or arterial application of angiogenic proteins, such as PDGF14,27 have been demonstrated to improve the survival of compromised flaps in various animal models, including the rat transverse rectus abdominis muscle (TRAM) myocutaneous flap. Herein, we report that prophylactic injection of collagen-embedded PDGF-B, but not FGF2, plasmid DNA markedly increases flap survival in a rat TRAM flap. Both plasmids stimulate an increase in the flap capillary network, but only PDGF-B plasmid DNA seems to promote sufficient perfusion for flap survival. The magnitude of the effect elicited by a 1-time injection of PDGF-B plasmid DNA in collagen before flap elevation suggests a promising clinical application of matrix-enabled nonviral gene transfer. The failure of FGF2 plasmid DNA to be effective despite histological evidence of angiogenesis suggests the importance of functional angiogenesis and the utility of this model in exploring mechanisms of angiogenesis.

RAT TRAM MODEL

Sprague-Dawley rats, weighing 230 to 300 g, were anesthetized with ketamine hydrochloride (87 mg/kg) and xylazine hydrochloride (13 mg/kg) via intraperitoneal injection. Their abdomens were shaved, scrubbed with iodine surgical soap, rinsed with sterile saline solution, and prepped with 10% povidone-iodine (Betadine) solution. The level of anesthesia was monitored throughout the procedure by observation of breathing pattern and response to pain (foot pad pinch). The xiphoid and symphysis pubis were used as constant landmarks, with the distance between them serving as the abdominal height. The flap extends 3 cm (cranial-caudal) and 3.5 cm bilaterally from the midline (total of 21 cm2). The borders of the skin paddle were incised down to the deep fascia. The skin was elevated on both sides to the borders of the left rectus abdominis muscle. The rectus abdominis muscle was then divided at the cephalic border of the flap and inferiorly from the posterior rectus sheath down to the symphysis pubis so that the inferior deep epigastric artery and vein served as the only vascular bundle serving the flap. A 3 x 7-cm silicone sheet was placed over the flap donor defect to prevent revascularization from the wound bed. The flap was sutured with 6-0 Vicryl to the original wound bed using an interrupted pattern. All procedures were approved by the Animal Care and Use Committee at Northwestern University.

At postoperative day 7, the total flap area and necrotic zones were traced onto a clear acrylic sheet placed over the flap. Visible tissue (ie, pliable, fur-bearing tissue) was calculated as a percentage of both original flap area and flap area at postoperative day 7, yielding similar results.

PLASMID CONSTRUCTION

Construction of the plasmid encoding the 109 amino acid form of human PDGF-B (pSG-PDGF-B) has been reported previously. The plasmid encoding the 18-kD form of human FGF2 (pSG-FGF2) was constructed by Selective Genetics (and is described in Doukas et al31). Both plasmids use the cytomegalovirus promoter to drive transgene expression, and have demonstrated activity in various in vitro and in vivo assays.31,32

PLASMID DNA-COLLAGEN MATRIX PREPARATION AND DELIVERY

Vectors were prepared in a 2% bovine type I collagen-gelatin formulation as previously described. Briefly, vectors were combined with monomeric bovine type I collagen (1.5 mg/mL; Celsion Technologies, Palo Alto, Calif), minimal essential medium at half strength (Life Technologies, Grand Island, NY), and 21.4 mmol/L of sodium bicarbonate (pH 7.4) to achieve final concentrations of 1.3, 3.0, or 6.0 mg plasmid DNA per milliliter matrix. The resultant mixture is an injectable homogeneous solution of plasmid DNA in the collagen matrix. For injections, the flap was divided into two 3 x 1.5-cm sections at the median plane. A 30-gauge, 2.54-cm needle was introduced subcutaneously at the center of each section toward each corner. While withdrawing the needle, 0.8 mL of the plasmid DNA-matrix mixture was evenly injected. This was repeated for the 4 corners of each section resulting in final delivery of 1.2, 2.4, or 4.8 mg plasmid DNA per flap (57, 114, or 228 mg/cm2, respectively).

TISSUE PROCESSING AND STAINING

After the rats were humanely killed, the anterior abdominal skin with the rectus muscle attached was dissected away from the other structures of the anterior abdominal wall. Tissue samples were isolated from the middle third of the flap, fixed with zinc-formalin, and processed for paraffin embedding. Five-micron cross sections were stained with hematoxylin-eosin or biotinylated Griffonia simplicifolia lectin-B4 (Vector Laboratories, Burlingame, Calif) for visualization of the capillary plexus within the flap tissue. For lectin staining, dewaxed sections were covered with 1% hydrogen peroxide for 5 minutes followed by overnight incubation with the isoelectin diluted in phosphate-buffered saline plus 1% bovine serum albumen (Sigma Chemical Co, St Louis, Mo) plus 0.1% Tween 20 plus 0.1 mmol/L calcium solution (Sigma Chemical Co) at room temperature. After washing, the sections were incubated with streptavidin–horseradish peroxidase (Vector Laboratories) for 20 minutes followed by treatment with diaminobenzidine (Vector Laboratories) until production of sufficient signal occurred at which time the reaction was stopped by rinsing the slide in distilled water.

TRANSDERMAL ORTHOGONAL POLARIZATION SPECTRAL IMAGING

Orthogonal polarization spectral imaging was performed using the CytoScan Imaging System (model E-II; Rheologics Inc, Exton, Penn) to determine if there were any differences in the microcirculation structure or perfusion in flaps treated with plasmid DNA transporting the PDGF-B or FGF2 transgenes. Flap skin received injections of matrix alone (control), FGF2, or PDGF-B plasmid DNA-matrix (4.8 mg per flap) as described.
Seven days after flap elevation, the animals were anesthetized and 3 locations on the viable portions of each flap were imaged by an operator (K.J.C.) blinded to treatment. The locations were chosen so that all were equidistant from the base of the pedicle and were obviously viable (ie, pliable, fur-bearing tissue without signs of necrosis). Based on these criteria, 17 images were collected from 7 control flaps, 19 images were collected from 8 FGF2-treated flaps, and 27 images were collected from 9 flaps treated with PDGF-B. Images were collected on S-VHS tape (Super Video Home System; Sony, New York, NY) and analyzed offline using a computer-assisted microcirculation analysis system (Capiscope; KK Technology, Holyford, Devon, England). Vascularization is indicative of the total number of vessels in a given image.

**STATISTICAL ANALYSIS**

Statistical analysis for pairwise comparisons was performed with unpaired t tests, with P<.05 indicating statistical significance. Group comparisons were made by 1-way analysis of variance with the Tukey Honestly Significant Difference test, when required.

### RESULTS

The vascular anatomical features of the rat TRAM are similar to those of the human, making the rat TRAM flap an approximate of the human version. An important difference is that the rat model is purposely compromised by division of the dominant circulatory feed vessels (ie, deep superior epigastric artery and vein) and placement of a silicone sheet between the flap and abdominal wall to reduce plasmatic imbibition and angiogenesis from the underlying pedicle.30 This design ensures severe ischemia, particularly at the flap edges, resulting in extensive necrosis (approximately 76%) 7 days after creation of the flap.

To determine the ability of PDGF-B to maintain viable tissue within the flap, increasing concentrations of collagen matrix–embedded PDGF-B plasmid DNA were injected into the skin to compose the flap 7 days before surgery. Platelet-derived growth factor B is a demonstrated promoter of angiogenesis in vivo and in vitro, and has been shown to improve survival and function of ischemic tissue in various in vivo models.14,33-35 In this model, PDGF-B plasmid DNA increased tissue viability in a concentration-dependent manner (Figure 1), with the highest dose tested (6.0 mg/mL of plasmid or 228 µg/cm²) resulting in a more than 2-fold increase in viable tissue compared with matrix alone (55% vs 24%; P<.01). The lowest concentration tested (1.5 mg/mL of plasmid or 57 µg/cm²) also achieved a statistically significant increase in viable tissue within the ischemic flaps (44% survival; P<.01 vs matrix alone). Tissue survival after the injection of 2% collagen alone (vehicle control) (Figure 1) or collagen containing 1.5 mg of plasmid DNA encoding the irrelevant reporter protein luciferase (plasmid control) was not significantly (P = .83) different from that of untreated flaps (data not shown).

Similar findings were observed following the injection of PDGF-B plasmid DNA 5 days before flap creation; however, positive effects were not seen when flaps were treated 3 days before surgery (data not shown), likely reflective of the time necessary for cellular infiltration of the matrix, plasmid uptake, and expression.36,37 Spindle-shaped cells and cordlike structures were observed histologically within the matrix (Figure 2), although the cell types involved in PDGF-B uptake and expression are not known. Reverse transcription–polymerase chain reaction analysis of skin samples injected with the PDGF-B plas-
mid DNA 7 days earlier confirms expression of the human PDGF-B message in the flap. Faint bands were consistently observed in control skin (treated with matrix alone), possibly from an endogenous signal induced by the matrix and/or the injection procedure that was detectable with the human primers. Sequence analysis confirmed the human origin of the PDGF-B message (data not shown).

Fibroblast growth factor 2 (or basic FGF) is another cytokine known to support therapeutic angiogenesis in vivo and angiogenic processes in vitro. We, therefore, tested FGF2 plasmid DNA (4.8 mg of DNA per flap) to determine its ability to support flap survival in the rat TRAM flap model. In an identical approach to that used with the PDGF-B plasmid DNA, matrix-embedded FGF2 plasmid DNA was injected into the skin that would compose the flap 7 days before flap elevation. Contrary to previous reports in other in vivo models, FGF2 did not support maintenance of ischemic tissue within the TRAM flap during the 7-day experimental period (Figure 3). However, compared with 2% collagen (Figure 4A and D), tissue analysis in flaps treated with PDGF-B and FGF2 plasmid DNA revealed an increase in tissue infiltration by cells (Figure 4B and C, respectively) and in lectin staining (Figure 4E and F, respectively) within the viable areas of the flaps. The histological findings suggest that the plasmid was actively expressed, which was confirmed by reverse transcription–polymerase chain reaction detection of the human FGF2 message in tissue samples, and resulted in an increase in the capillary network within the flaps because lectin specifically labels capillary endothelial cells. The differential effects of PDGF-B and FGF2 on flap survival, despite similar lectin-staining patterns, suggest that other critical processes are involved in the ultimate survival of ischemic tissue beyond the histological presence of a capillary network.

To further address this issue, flaps injected with matrix alone, PDGF-B plasmid, or FGF2 plasmid were subjected to orthogonal polarization spectral imaging (Cytoscan). This noninvasive technique allows still-image visualization and analysis of blood vessels within tissue dependent on the cellular motion associated with perfusion and has been demonstrated to correlate strongly with fluorescent microscopic imaging. Measurements were averaged from 3 different locations on each flap 3 days after flap elevation. This time point was chosen to obtain measurements before the onset of any excessive necrosis across the flap. Computer image analysis revealed a significant increase in vascularity in PDGF-B plasmid-injected flaps compared with either FGF2 plasmid– or matrix-treated flaps ($P<.03$) (Figure 5). Therefore, the inability of FGF2 plasmid treatment to support flap viability, despite the enhanced capillary network, may in part be due to the insufficient perfusion of the neovasculature.
Ischemia negatively affects normal tissue function and when, excessive or prolonged, is a causative factor of cellular apoptosis/necrosis associated with such pathological features as myocardial infarction, stroke, and major wounds. Treatment strategies are focused on normalizing local blood flow, with the end goal of restoring function to and/or preventing loss of function in ischemic tissue. Approaches in practice include surgical procedures, such as coronary artery bypass grafting and lower limb revascularization procedures designed to alleviate tissue ischemia through reestablishment of normal circulation upstream of the microvasculature. The success of such approaches is then partly limited by the microcirculatory perfusion area within the ischemic tissue. By inference, any increase in the perfusion area in combination with sufficient circulatory feed will prevent ischemic tissue damage and necrosis. This is the principle behind therapeutic angiogenesis.

Herein, we demonstrate that the prophylactic injection of rat TRAM flaps with collagen matrix–embedded PDGF-B plasmid prevented flap necrosis. While the vascular anatomical features of the rat TRAM flap are similar to those of the human version, with 2 pairs of feed vessels (the deep inferior and superior epigastric arteries and veins), the rat model differs from the standard human TRAM flap in that it is based on the nondominant (inferior) pair for circulatory support of the flap tissue. This results in profound ischemia and a low degree of flap survival in the control groups (approximately 24%). Injection of the PDGF-B plasmid DNA 1 week before flap creation increased maintenance of viable tissue up to 2.4-fold greater than control (matrix alone) injections. The response was concentration dependent, with significant increases in survival observed with as little as 37 µg of plasmid per square centimeter of tissue. Our findings are in agreement with those of previous reports demonstrating the efficacy of PDGF-B, in either recombinant protein form or plasmid form, for promoting the survival of ischemic tissue and flaps. However, the magnitude of the effect in a clinically relevant model with a plasmid DNA suggests the potential for translation into the clinical arena.

Surprisingly, treatment of the ischemic flaps with FGF2 (or basic FGF) plasmid DNA resulted in no observed increase in flap survival, despite having a similar outcome on the capillary network to PDGF-B. While differences in the level of bioactive protein could possibly explain the observed difference between PDGF-B and FGF2 plasmid DNA, the lectin staining demonstrated an active tissue response to both transgene products within the experimental time frame. Although FGF2 has been demonstrated to promote angiogenesis and support tissue viability in various in vivo models, several reports share similarities with our findings. In a rat model of coronary occlusion, Inagaki et al reported increased coronary angiogenesis without resultant changes in blood flow or necrosis after treatment with recombinant FGF2. Differential results between proangiogenic cytokines have also been observed in other models of ischemia-compromised tissue. In an ex vivo gene therapy approach, myoblasts producing FGF2 or variants of vascular endothelial cell growth factor were transplanted into a rat model of a random pattern ischemic skin flap. Flaps embedded with FGF2-producing cells displayed increased neovascularization and survival compared with flaps embedded with cells producing vascular endothelial cell growth factor 121 or 166. Taken together, our data further emphasize that establishment of a capillary network is required, but may not be sufficient, for tissue survival in the face of ischemia and that tissue function should be a key outcome measurement in such experiments. The concept of functional angiogenesis is important, and this model offers a reliable way to compare the impact of different potential therapeutic angiogenic factors.

In vivo, PDGF-B and FGF2 are pluripotent cytokines stimulating monocyte infiltration and mesenchymal cell proliferation and migration. The findings reported herein suggest both support capillary network formation, as evidenced by the increase in lectin staining over control tissue, suggesting that perfusion of that network differs sufficiently to affect survival of cutaneous flap tissue. This theory was supported by spectral scanning of PDGF-B and FGF2 plasmid DNA–treated flaps before the gross appearance of necrosis. Both cytokines are vasoactive, and both have been reported to influence either angiogenesis or arterial repair. Compared with FGF2 in this model, PDGF-B seems capable of maintaining or stimulating sufficient blood flow to prevent tissue necrosis after the creation of the compromised flap. The exact point of action of PDGF-B is unknown, but may include modulation of local vascular control mechanisms.

In conclusion, our results support the use of matrix-enabled plasmid gene transfer–driven angiogenesis to support survival and function of ischemia-challenged tissues and indicate that prophylactic therapy may be useful for the treatment of tissues, like surgical flaps, that will potentially be impacted by such a challenge. Our results further support the careful consideration of transgene, tissue composition, and vascular network as important variables in the treatment of ischemic tissue.

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REFERENCES


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We face the challenges common to all major academic institutions: declining revenues, soaring malpractice costs, and restrictions on our ability to educate residents. We remain steadfast in our commitment to excellence in clinical service, clinical innovation, education, and research. As the only major academic medical institution in the region, we are uniquely positioned to meet these challenges independent of local competing forces.

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Correction

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