Stimulation of Angiogenesis to Improve the Viability of Prefabricated Flaps

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The cutaneous area in a prefabricated myocutaneous flap surviving after elevation is dependent on the rate and amount of vascular ingrowth that occurs from the underlying muscle. Two modalities, basic fibroblast growth factor and hyperbaric oxygen, were used separately and together in a prefabricated myocutaneous flap animal model to improve flap survival. The semimembranous muscle, based on the saphenous vessels of 40 female Wistar rats weighing between 250 and 325 grams, was tunneled under the ipsilateral abdominal skin and sutured in place. A 3 × 5-cm silicone sheet was placed beneath the muscle flap, and the ipsilateral epigastric vessels were ligated. Four groups of 10 animals each received one of the following treatment regimes: a 1-ml normal saline infusion into the saphenous arterial pedicle, a 1-ml infusion of basic fibroblast growth factor (1.0 μg/gm of muscle), a 1-ml normal saline infusion and 14 hyperbaric oxygen treatments, or a 1-ml basic fibroblast growth factor infusion and 14 hyperbaric oxygen treatments. After 1 week, the muscle, still based on the saphenous vessels, was elevated with a 3 × 5-cm abdominal skin paddle. The flap was sutured back in place, leaving the silicone sheet intact. The surviving area of each flap was measured 1 week later after it had demarcated into viable and necrotic regions. Laser Doppler skin perfusion measurements were taken before and after flap elevation and before animal euthanasia. Sixteen flaps, 4 in each group, were examined histologically for vascularity by means of hematoxylin and eosin staining. There was a statistically significant increase in flap survival area when either basic fibroblast growth factor or hyperbaric oxygen was used alone. Further improvement was noted with combination therapy. Histology confirmed improved vascularity in the basic fibroblast growth factor and hyperbaric oxygen-treated flaps. This study shows a significant and reliable increase in the area of prefabricated myocutaneous flap survival using either basic fibroblast growth factor or hyperbaric oxygen. There is a further complementary effect when these two modalities are combined, leading to near complete flap survival through improved vascularity. (*Plast. Reconstr. Surg.* 101: 1290, 1998.)

The availability of flap donor sites is limited by the intrinsic vascular architecture of each flap. The conventional flaps available do not always satisfy the unique requirements of a specific recipient site. Flap prefabrication from a transferred vascular pedicle is a recognized method of capturing an existing skin territory that could not otherwise be elevated on a single vascular pedicle. Other methods such as surgical delay, tissue expansion, hyperbaric oxygen, and topical growth factors have all shown promise in improving the skin vascularity in prefabricated flaps. This study investigated the potential angiogenic properties of basic fibroblast growth factor and hyperbaric oxygen in a prefabricated myocutaneous flap model to assess the possibility of improving the area of flap survival. The combination of basic fibroblast growth factor and hyperbaric oxygen was further examined for a possible potentiating effect.

Methods

Forty female Wistar rats weighing between 225 and 275 grams were used. Sex and weight-adjusted doses of Nembutal (45 mg/kg, IP) were used to anesthetize the animals. All surgeries were performed under sterile conditions and in accordance with the guidelines of the Animal Research Committee of our institution. The abdomen and the right leg were shaved in each animal. The semimembranous muscle in the lower extremity was exposed through a longitudinal 2.5 to 3.0-cm incision in the medial aspect of the right leg (Fig. 1, *above*). The saphenous vascular pedicle was identified using the operating microscope and isolated from its insertion into the muscle up to the femoral vessels. A 3 × 5-cm pocket was dissected under the ipsilateral portion of the an-

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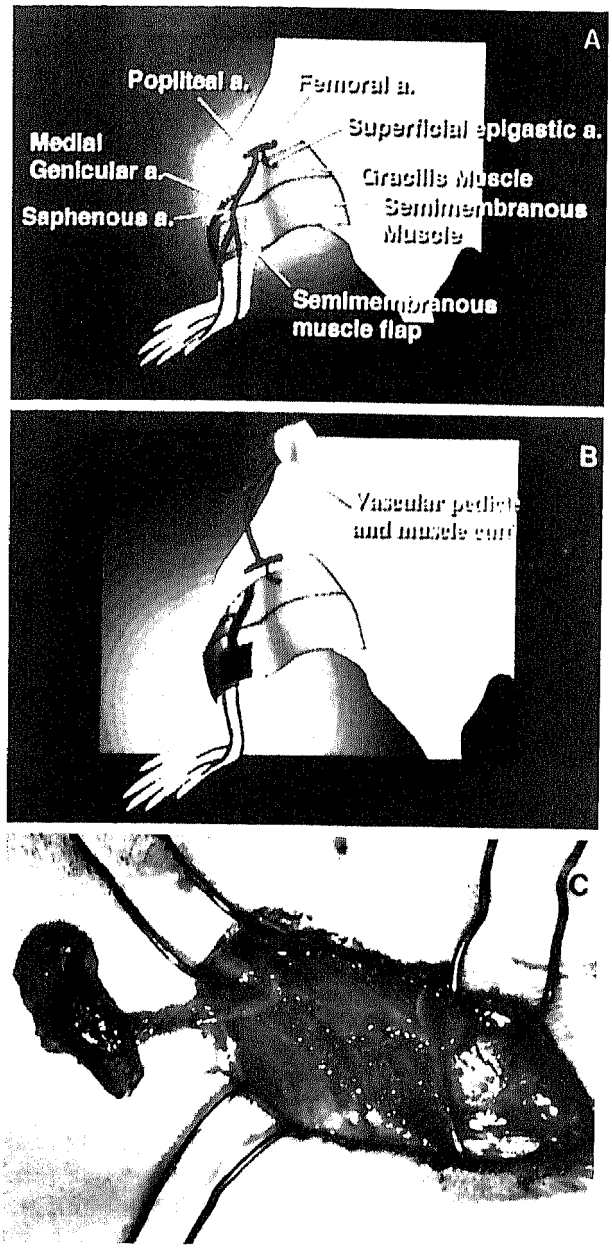

FIG. 1. (*Above*) Schematic representation of the rat semimembranous muscle on the saphenous pedicle. (*Center*) Rotation of the semimembranous muscle on the saphenous pedicle beneath the anterior abdominal border of the epigastric skin flap. (*Below*) The actual semimembranous muscle isolated on the vascular pedicle.

terior abdominal wall, beneath the panniculus carnosus. A 10 × 15-mm piece of the semimembranous muscle was tunneled under the anterior abdominal wall into the pocket, where it was sutured into position (Fig. 1, *center* and *below*). A 3 × 5-cm silicone sheet (Applied Biomaterial Technologies) 0.015 inches thick was placed under the muscle to prevent vascularization from the bed. The ipsilateral superficial epigastric artery was ligated to render this portion of the abdominal wall relatively ischemic as a stimulus for neovascularization. Polyethylene tubing (0.28 mm) was fed into the femoral artery and placed at the takeoff of the saphenous artery. All animals received a 1-ml infusion using a Harvard Apparatus IV syringe pump. The control group (n = 10) received a 1-ml infusion of normal saline. The hyperbaric oxygen group (n = 10) received the same saline infusion followed by hyperbaric oxygen treatments. Each treatment lasted for 90 minutes at 2.5 atmospheric pressure of 100% oxygen every 12 hours, for a total of 14 treatments over a 7-day period. The growth factor group (n = 10) received an infusion of 1 μg of basic fibroblast growth factor per gram of muscle tissue (Sigma). The combination treatment group (n = 10) received the above infusion of basic fibroblast growth factor followed by 14 hyperbaric oxygen treatments over the following week.

The animals were again anesthetized 1 week after the initial procedure. A 3 × 5-cm abdominal wall flap that incorporated the semimembranous muscle was elevated based on the saphenous vessels, and the silicone sheet was undisturbed. The prefabricated myocutaneous flap was sutured back in place, and laser Doppler perfusion measurements of the distal flap were taken. The reliability of the laser Doppler readings from animal-to-animal and day-to-day was assured by uniform skin surface preparation, consistent probe pressure on the skin, and frequent zero calibration checks. The animals were observed for up to 7 days to allow all flaps to demarcate into viable and nonviable regions as determined by color, texture, and laser Doppler perfusion readings. Flap necrosis or survival was complete by the seventh postoperative day. The surface area of the flap that survived was measured using a paper trace from each flap; the paper trace was then digitally scanned, and the area was quantitated with digital planimetry. The flaps were then harvested, and the animals were euthanized using a 1-ml intracardiac injection of lidocaine. The harvested flaps were preserved in 10% formalin solution, sectioned, and stained with hematoxylin-eosin for histologic analysis. The vascularity of the most distal viable portion of the flaps was analyzed under 40× magnification counting the number of vessels per high power field in four flaps from each group.

The average area of flap survival, distal laser Doppler perfusion after elevation, and the

number of vessels observed per high power field were compared between groups. Statistical analysis using a one way analysis of variance and post hoc Tukey all-pairwise multiple comparison procedure for individual differences were performed with $p < 0.05$ considered statistically significant.

Results

Two rats cannibalized their flaps before demarcation and were excluded from the study. The following data are based on the remaining 38 animals.

Flap Survival

The planimetric analysis of flap survival areas is shown in Figure 2 (*above*). A statistically significant improvement in the average area of flap survival was observed in the hyperbaric oxygen group (5.5 ± 0.86 cm^2, mean ± SD) and basic fibroblast growth factor group (8.3 ± 1.29 cm^2) relative to the control group (1.6 ± 0.81 cm^2). The average area of flap survival in animals receiving combination therapy with hyperbaric oxygen + basic fibroblast growth factor (9.7 ± 0.96 cm^2) was significantly better than the controls or either treatment alone. The results listed as percent survival area are 10.6, 36.7, 55.3, and 64.6 percent survival for normal saline, normal saline + hyperbaric oxygen, basic fibroblast growth factor, and basic fibroblast growth factor + hyperbaric oxygen groups, respectively.

Laser Doppler

Laser Doppler readings in distal areas of the prefabricated flaps taken immediately after elevation showed consistently higher, but not statistically significant, readings in the flaps that received either basic fibroblast growth factor or hyperbaric oxygen when compared with the control normal saline group (Fig. 2, *center*). The combination of basic fibroblast growth factor and hyperbaric oxygen resulted in laser Doppler perfusion in the distal portion of the flap that was greater than the control, hyperbaric oxygen-, or basic fibroblast growth factor alone-treated animals. These differences were statistically significant.

Histology

The histology of the most distal viable areas of the flaps was analyzed in four animals from each group for the number of vessels present per high power field as a direct measurement

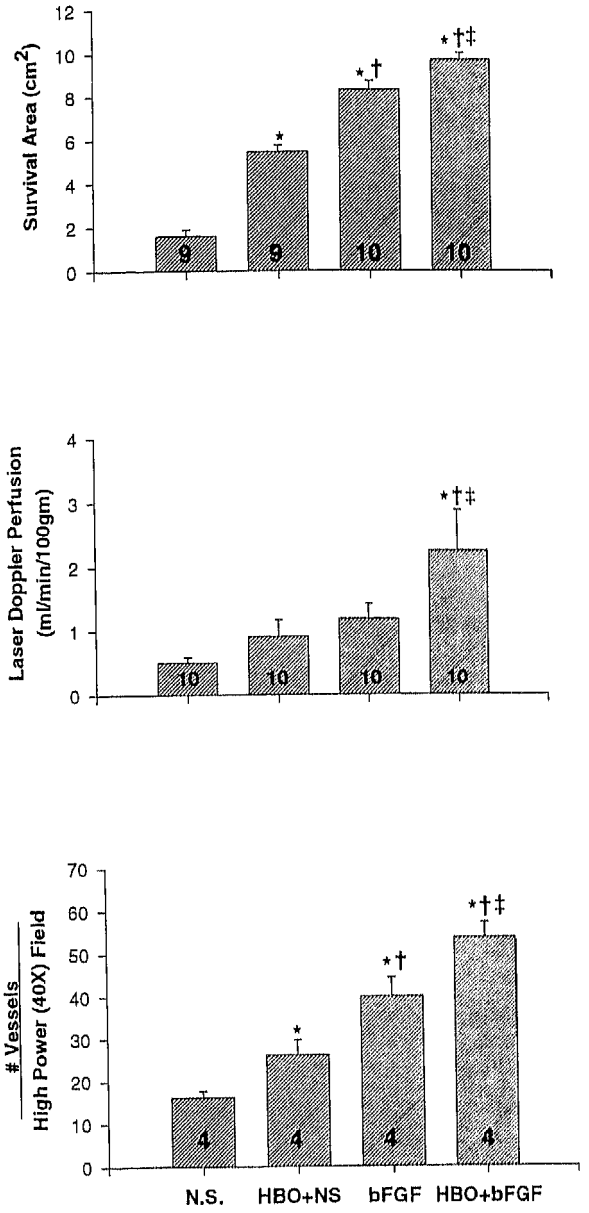

FIG. 2. (*Above*) The average survival area for saline control flaps ($n = 9$) was less than those treated with hyperbaric oxygen and infused with normal saline ($n = 9$). The flaps treated with basic fibroblast growth factor ($n = 10$) had significantly greater survival than both the hyperbaric oxygen and saline flaps. The flaps receiving combination therapy with both hyperbaric oxygen and basic fibroblast growth factor had significantly greater survival than all other flaps (*, $p < 0.05$ versus saline; †, $p < 0.05$ versus hyperbaric oxygen; ‡, $p < 0.05$ versus basic fibroblast growth factor). Values shown are means with error bars equal to SEM. (*Center*) Laser Doppler perfusion measured in the distal epigastric flap after elevation (*, $p < 0.05$ versus saline; †, $p < 0.05$ versus hyperbaric oxygen; ‡, $p < 0.05$ versus basic fibroblast growth factor). Values shown are means with error bars equal to SEM. (*Below*) Microvessels counted in histologic sections of the most distal portion of surviving skin flaps measured 1 week following elevation (*, $p < 0.05$ versus saline; †, $p < 0.05$ versus hyperbaric oxygen; ‡, $p < 0.05$ versus basic fibroblast growth factor). Values shown are means with error bars equal to SEM.

of neovascularization. The lowest-to-highest number of vessels present in the surviving distal third of the flap were seen in the following order: normal saline, hyperbaric oxygen, basic fibroblast growth factor, and basic fibroblast growth factor + hyperbaric oxygen (Fig. 2, *below*). These results correlate well with the improvement seen in flap survival (Fig. 2, *above*).

Discussion

For years, surgeons have recognized a need to create custom-made flaps that meet the special requirements of a given recipient site. Prefabrication is one method of creating specialized flaps from a donor site that would not otherwise be available on a single vascular pedicle. The next step is to improve the reliability and survival characteristics of these specialized flaps.

Flap prefabrication dates to 1971 when Washio[1] used a segment of vascularized ileum brought onto the abdomen to revascularize the overlying subcutaneous tissue and skin, which subsequently survived as a composite free flap transfer. Subsequently, other investigators[2] have devised prefabricated models to include skin, muscle, cartilage, bone, and even an entire knee joint in a rat model. Investigators have attempted to manipulate the physiology and microanatomy of prefabricated flaps to improve their vascularity and viability. These attempts have included surgical delay,[3] tissue expansion,[4] and application of tumor growth factor-β.[5] Homma et al.[4] demonstrated a larger area of flap survival in expanded muscle vascularized pedicle flaps than were found in non-expanded flaps in rats. Topical application of tumor growth factor-β in a prefabricated fasciocutaneous flap led to an accelerated rate of maturation in these flaps, but no long-term increase in flap survival area was found. Gospodarowicz[6] and Scweigerer[7] elucidated the biochemical properties of basic fibroblast growth factor as a potent angiogenic growth factor. The angiogenic action of basic fibroblast growth factor led to a reduction of infarct size in ischemic myocardium in a dog model.[8] Basic fibroblast growth factor binds to the heparin-sulfate proteoglycan in the extracellular matrix, which serves as a tissue reservoir from where it exerts its angiogenic action.

Previous growth factor studies have employed only topical routes of administration. Topical application is not a reliable method to deliver a specific dose of the drug because free growth factor has a short half-life when it is not bound by the vascular endothelium and heparin sulfate in the interstitial space. We chose the intra-arterial infusion route to increase drug delivery into the transferred semimembranous muscle microcirculation and interstitial space. The half-life of bound basic fibroblast growth factor is several days. It was postulated that drug binding in the muscle extracellular matrix might eliminate the problems with unreliable delivery and growth factor washout. The improvement in the area of skin capture by the transferred muscle could therefore be attributed to the prolonged effect of basic fibroblast growth factor. Histologic studies confirmed a significant increase in the number of vessels in the basic fibroblast growth factor-treated flaps. Thus, basic fibroblast growth factor probably improved the area of survival in this prefabricated myocutaneous flap model through neovascularization. We did not differentiate arteries from veins to determine which kind of vessel was most prominent in each group nor was vessel caliber analyzed.

There has been a lot of controversy and investigation regarding the angiogenic properties of hyperbaric oxygen. In skin wounds, hyperbaric oxygen was found to increase the bursting strength and to stimulate angiogenesis histologically.[9] Manson et al.[10] showed that capillaries grew almost three times further distally into guinea pig pedicled flaps in animals that were treated with hyperbaric oxygen when compared with age-matched controls.

Neovascularization has also been noted in several clinical situations where hyperbaric oxygen therapy was employed. An increased vascularity in postradiated tissue following hyperbaric oxygen therapy has been documented clinically in cases of head and neck cancer after resection and radiation therapy.[11]

In this study, hyperbaric oxygen therapy alone led to improved survival as well as increased vascularity in the prefabricated flaps. However, basic fibroblast growth factor alone was more effective than hyperbaric oxygen. The combination of basic fibroblast growth factor and hyperbaric oxygen, interestingly, improved survival above and beyond the effect of either agent alone, suggesting these agents potentiate one another. This hypothesis was further substantiated in the histologic sections demonstrating the highest number of vascular elements in the combination group. Near com-

plete survival of the prefabricated myocutaneous flaps were achieved in the combined hyperbaric oxygen and basic fibroblast growth factor-treated animals. A previous study by Zhao et al.[12] showed a similar additive effect on angiogenesis by combining hyperbaric oxygen and several different growth factors in a rabbit ischemic ulcer model.

Many factors influence flap vascularity.[4,5,10] Increased vascularity may involve one or more of three possible processes. The first process is inosculation where capillaries of the recipient bed and those of the flap join together to reestablish antegrade flow. The other two processes are subtypes of neovascularization. Neovascularization can originate from capillary sprouting from preexisting vessels within a flap increasing the absolute number of vessels in a given area. Alternatively, recipient-bed vessels can grow into the flap. The difference between these types of neovascularization is that the "mother" vessels are of different origins, one indigenous to the flap, the other indigenous to the recipient bed. It is reasonable that there is a common internal pathway of new vessel growth irrespective of the ultimate process responsible for enhancing vascularity. This poorly understood concept, called angiogenesis, precedes inosculating revascularization and is different from neovascularization. Angiogenesis and neovascularization are likely a continuum of the same biologic process that may be fostered in the local environment by angiogenic factors such as platelet-derived growth factor, tumor growth factor, and basic fibroblast growth factor or by regional factors such as global or distal ischemia. Examining the number of vessels in each high power field was deemed appropriate to qualitatively and quantitatively measure the absolute vascularity in an objective manner.[13] Other methods of measuring vascularity, such as xenon clearance and infrared thermography, do not quantitate the amount of neovascularization and/or angiogenesis.[14–16] The increase in the number of vessels identified per high power field in the hyperbaric oxygen, basic fibroblast growth factor, and hyperbaric oxygen + basic fibroblast growth factor groups (Fig. 2, *below*) were thought to be evidence of the angiogenesis and/or neovascularization process. This implies that manipulation of the local environment by the addition of hyperbaric oxygen and/or basic fibroblast growth factor stimulates this yet to be defined pathway.

This study has important clinical implications. The angiogenic properties of hyperbaric oxygen or basic fibroblast growth factor, alone or in combination, could be applied to many clinical situations where partial flap loss or a tenuous blood supply is of concern. We have shown that infusion of basic fibroblast growth factor is a reliable method of local drug delivery in a pedicled flap model. Partial flap ischemia in the random distal portions of some myocutaneous flaps may lead to distal flap necrosis. The results of this study might be applied to increase the vascularity in the random portion of an axial pattern flap before elevation through neovascularization or improve the survival of "custom-made" flaps.

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REFERENCES

1. Washio, H. An intestinal conduit for free transplantation of other tissues. *Plast. Reconstr. Surg.* 48: 48, 1971.
2. Khouri, R. K., Upton, J., and Shaw, W. W. Prefabrication of composite free flaps through staged microvascular transfer: An experimental and clinical study (see comments). *Plast. Reconstr. Surg.* 87: 108, 1991.
3. Tark, K. C., Khouri, R. K., Shin, K. S., and Shaw, W. W. The fasciovascular pedicle for revascularization of other tissues. *Ann. Plast. Surg.* 26: 149, 1991.
4. Homma, K., Ohura, T., Sugihara, T., Yoshida, T., and Hasegawa, T. Prefabricated flaps using tissue expanders: An experimental study in rats. *Plast. Reconstr. Surg.* 91: 1098, 1993.
5. Iwasawa, M. Accelerated maturation in prefabricated flaps by transforming growth factor-beta: An experimental study in the rabbit. *Ann. Plast. Surg.* 31: 72, 1993.
6. Gospodarowicz, D. Fibroblast growth factors: From genes to clinical applications. *Cell Biol. Rev.* 25: 307, 1991.
7. Schweigerer, L. Basic fibroblast growth factor and its relation to angiogenesis in normal and neoplastic tissue. *Klin. Wochenschr.* 66: 340, 1988.
8. Yanagisawa-Miwa, A., Uchida, Y., Nakamura, F., et al. Salvage of infarcted myocardium by angiogenic action of basic fibroblast growth factor. *Science* 257: 1401, 1992.
9. Meltzer, T., and Myers, B. The effect of hyperbaric oxygen on the bursting strength and rate of vascularization of skin wounds in the rat. *Am. Surg.* 52: 659, 1986.
10. Manson, P. N., Im, M. J., Myers, R. A. M., and Hoopes, J. E. Improved capillaries by hyperbaric oxygen in skin flaps. *Surg. Forum* 31: 564, 1980.
11. Myers, R. A., and Marx, R. E. Use of hyperbaric oxygen in postradiation head and neck surgery. *NCI Monogr.* 151:7, 1990.

12. Zhao, L. L., Davidson, J. D., Wee, S. C., Roth, S. I., and Mustoe, T. A. Effect of hyperbaric oxygen and growth factors on rabbit ear ischemic ulcers. *Arch. Surg.* 129: 1043, 1994.
13. Hu, D. E., Hiley, C. R., Smither, R. L., Gresham, G. A., and Fan, T. P. Correlation of 133Xe clearance, blood flow and histology in the rat sponge model for angiogenesis: Further studies with angiogenic modifiers. *Lab. Invest.* 72: 601, 1995.
14. Meyer, G. T., and McGeachie, J. K. Angiogenesis in the developing corpus luteum of pregnant rats: A stereologic and autoradiographic study. *Anat. Rec.* 222: 18, 1988.
15. White, B. A., Lockhart, P. B., Connolly, S. F., and Sonis, S. T. The use of infrared thermography in the evaluation of oral lesions. *J. Am. Dent. Assoc.* 113: 783, 1986.
16. Eriksdotter-Nilsson, M., Bjorklund, H., and Olson, L. Laminin immunohistochemistry: A simple method to visualize and quantitate vascular structures in the mammalian brain. *J. Neurosci. Methods* 17: 275, 1986.