Recombinant Leptin Administration Improves Early Angiogenesis in Full-thickness Skin Flaps: An Experimental Study

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Abstract. Background: Leptin is a potent direct angiogenic factor that stimulates endothelial cell migration and activation in vitro, and angiogenesis in vivo. In addition, leptin seems to play an important role in angiogenesis as it promotes the formation of new blood vessels. Objective: To determine the effect of local application of exogenous leptin on the survival of full thickness skin flaps in an experimental animal model. Materials and Methods: Ninety Sprague-Dawley rats were used in this study. A full thickness dorsal flap (10 cm x 2 cm) with the pedicle located at the level of the iliac crest was designed. Animals were divided into ten groups of nine animals each. In the distal two thirds of the flap and by means of subdermal injection at 8 different locations, rats were injected with 100 ng/ml leptin, 250 ng/ml leptin, 500 ng/ml leptin, 1000 ng/ml leptin (groups A, B, C and D), 1 µg/ml VEGF (group E), or 1 ml saline (control group), respectively. For each of the four leptin doses used, another animal group was injected with a combination of leptin/antileptin: 100 ng/ml leptin with 150 ng/ml antileptin, 250 ng/ml leptin with 375 ng/ml antileptin, 500 ng/ml leptin with 750 ng/ml antileptin or 1000 ng/ml leptin with 1500 ng/ml antileptin (groups A1, B1, C1 and D1, respectively), in order to study the inhibition of the leptin factor. Nine rats served as controls and were injected with 1 ml saline solution. Rats were sacrificed 3, 7 and 9 days postoperatively. After sacrifice of the animals, the skin was grossly arranged on its appearance, colour and texture. Full thickness skin flaps were dissected for histological examination. A qualitative analysis of angiogenesis in the flap was conducted following a standard hematoxylin and eosin stain. The wound tissue samples from each experimental group underwent immunohistochemical evaluation of microvessel density by endothelial cell staining with mouse anti-rat CD 34 monoclonal antibody. Results: Immunohistochemical staining revealed that more granulation tissue and improved angiogenesis were observed in group D (1000 ng/ml leptin) flaps compared to those in the VEGF, leptin/antileptin and saline groups. In addition, skin flap survival rate in group D (1000 ng/ml leptin) and group E (1 µg/ml VEGF) were significantly better than those of the other groups. The most impressive formation of new blood vessels was noted in the groups with the higher leptin doses. Surgical wounds in the control, as well as in the leptin/antileptin groups, did not demonstrate any new vessels. Conclusion: Exogenous administration of recombinant leptin increases early skin flap angiogenesis in an experimental animal model. Local application of leptin could efficiently improve survival of ischemic skin flaps.

Partial necrosis of pedicle skin flaps remains a significant problem in plastic and reconstructive surgery. The causes of necrosis are inadequate arterial inflow, insufficient venous outflow, or both (1, 2). These factors lead to ischemia that frequently occurs in the portion of the flap lying most distally to the pedicle. Experimental work has shown that augmenting the vascularity of an ischemic skin flap can significantly improve flap survival (3, 4). Angiogenesis, one biological mechanism for the formation of new capillaries from pre-existing venules, is fundamental to wound healing and to the maintenance and repair of the vasculature (5).
The new blood vessels can be induced to invade ischemic muscle and skin flaps by ischemia itself (6, 7) and angiogenesis may be the mechanism of improving skin flap survival when using the delay procedure (8, 9). The potential of therapeutic agents, including a variety of growth factors, to stimulate the development of angiogenesis in ischemic skin flaps has recently aroused considerable interest (10-12). Consequently, intense experimental work is now focused on the pharmacological application of angiogenic growth factors in the compromised wound (13-15).

Leptin is a potent direct angiogenic factor that stimulates endothelial cell migration and activation in vitro, and angiogenesis, in vivo (16-22). The observation that leptin mediates angiogenic and mitogenic effects in vitro further implicates an important role for leptin as a mitogenic factor during tissue regeneration in vivo. In addition, leptin seems to play an important role in angiogenesis by directly promoting the formation of new blood vessels (20, 23).

This study aims to evaluate the effect of exogenously administered recombinant leptin on skin flap angiogenesis, in an experimental animal model.

Materials and Methods

Animals. Ninety Sprague-Dawley rats, weighing between 200-280 g, were used for the study. They were obtained from the Department of Physiology (University of Thrace, Alexandroupolis, Greece) and maintained under a 12-hour light/dark cycle at 22 °C until they were 30 weeks of age. At this time they were caged individually, allowed food and water ad libitum and monitored for body weight. This project was conducted at the Department of Experimental Surgery, Medical School, University of Thrace, Greece, and was supervised by the University’s veterinarian. The Veterinary Administration Medical Center, Alexandroupolis, Greece, approved the study protocol.

Preparation of skin tissues. Animals were divided into ten groups of nine rats each. After induction of general anesthesia with ether and ketamine 10 ml/kg (30 mg/kg), the dorsal regions were shaved, and the animals were placed in a prone position. Under aseptic conditions, a caudally based rectangular dorsal flap, measuring 2x10 cm², was symmetrically raised (24). The distal edge of the flap was situated 4 mm caudally to the lower edges of the scapulae. The flap consisted of skin, panniculus carnosus muscle, and submuscular areolar tissue. In this flap model, two arteries were constantly located at the flap’s base and were left intact. In the distal two thirds of the flap and by means of subdermal injection at 8 different locations, rats were injected with 100 ng/ml leptin (Cytolab Ltd. / Pepro Tech Asia, Rehovot, Israel) (group A), 250 ng/ml leptin (group B), 500 ng/ml leptin (group C), 1000 ng/ml leptin (group D), 1 µg/ml VEGF (vascular endothelial growth factor, Cytolab Ltd. / Pepro Tech Asia) (group E), or 1 ml saline (control group), respectively. For each of the four leptin doses used, another animal group was injected with a combination of leptin with antileptin (Cytolab Ltd. / Pepro Tech Asia): group A1: 100 ng/ml leptin with 150 ng/ml antileptin; group B1: 250 ng/ml leptin with 375 ng/ml antileptin; group C1: 500 ng/ml leptin with 750 ng/ml antileptin; group D1: 1000 ng/ml leptin with 1500 ng/ml antileptin, in order to study the inhibition of the leptin factor. Every flap was then returned to its bed and carefully sutured into place using 4/0 silk suture.

Animals were measured for body weight both before and after the end of the experiment, were kept in individual cages and sacrificed 3, 7 and 9 days after surgery.

Histological and immunohistochemical evaluation. Skin flaps in full thickness with subcutaneous tissues from the most distal and viable portion were dissected for histological examination. The specimens were fixed in 10% formalin, stored at 4°C and sectioned longitudinally at 2 µm thickness. A qualitative analysis of angiogenesis in the wound was performed following a standard hematoxylin and cosin (HE) stain.

Further analysis was performed by immunohistochemical evaluation. Surface endothelial cells were detected by a monoclonal anti-mouse anti-rat CD 34 antibody (Innovex Biosciences, CA, USA; dilution, 1:40). The slides were incubated in microwave oven at 600 W for 15 min. The immunohistological stain was performed on 2 µm paraffin sections and the specimens were embedded in Super Frost Plus Tissue Tek (Dako Corporation, Carpenteria, CA, USA). The sections were cleaned and were put in a microwave oven in Trilogy liquid. After cleaning with H2O2 3% and sterilized water, the sections were incubated with EnVision-kit (Dako Corporation, Carpenteria) for 25 min. They were then incubated with diaminobenzidine (DAB) for 15 min and washed with PBS for 5 min. The sections were dehydrated through a series of alcohol solutions.

The incubation was performed at a temperature of 37°C in order to increase the sensitivity of the stain. This method improves the immunohistological expression, decreases non-specific staining and allows higher dilution of the initial antibodies (25-28). A control set used histological sections with known positivity to the antibodies used.

The wound tissue samples from each experimental group underwent evaluation of the level of microvessel density (MVD) by measuring the number of capillaries per 15 high power fields. All endothelial cells were stained with anti-rat CD 34 antibody. Microvessels were represented by brown capillaries (Figure 1). Optical micrographs were taken under a high-power microscope (x400).

Statistical analysis. Statistical analysis was performed using the ANOVA test. A two-tailed unpaired Student’s t-test and an analysis of variance was used to analyze differences between groups. Comparison was made both between leptin and leptin/antileptin-treated or control groups. A p-value less than 0.05 was considered statistically significant.

Results

From gross examination, the surface of the flaps healed without any complications in both experimental and control groups both at 7 and 9 days, postoperatively. On day 7 after surgery, the survival regions were clearly demarcated from necrotic regions in every flap. The surviving skin flap appeared tender and normal in its texture and bled upon cutting. In contrast, the necrotic skin was black, rigid and did not bleed upon cutting. The distal portion of the flap in
the 1000 ng/ml leptin group and the VEGF group was much more alive and fresh appearing than those of the other groups (Figure 2).

It was found that skin flaps group D, treated with 1000 ng/ml leptin, and group E, treated with 1 μg/ml VEGF, had the greatest number of new vessels in comparison to the
other eight groups. The third best leptin group performance in regard to angiogenesis was that of group C where skin flaps were treated with 500 ng/ml of leptin.

The greatest formation of new vessels for all 4 doses of leptin was apparent seven days after injection. There was growth of inflammatory granulous tissue and angiogenesis focì around the lesion, hyperplasia of squamous epithelium and an increase, fattening and irregular distribution of collagen fibers. The semi-quantitative assessment of MVD (mean±SD) showed a statistically significant difference in wound repair between different doses of leptin at different postoperative days. However, there were no significant differences in MVD among the leptin treated groups at 7 and 9 days, postoperatively.

No wounds from the control group and those injected with the combination of leptin and antileptin exhibited formation of new blood vessels. There was however growth of inflammatory granulous tissue, with inflammatory cells of mixed type, fibrinoblastcells and fibrins of collagen instead (Table I). Most of the wounds at the third postoperative day for all four different doses of leptin had growth of inflammatory granulous tissue, fibrinoblastcells and fibrins of collagen, and formation of new vessels was apparent particularly at the edges of the skin flaps (Table I). There was broad development of fibroblasts focused toward the center with no signs of necrosis (Table I). Nine days after treatment for all four different doses of leptin, there was marked hyperplasia of squamous epithelium and an increase, fattening and irregular distribution of the collagen fibers. There was also broad appearance of fibroblasts focused towards the center with no signs of necrosis. The mean number of the blood vessels developed at nine days after treatment for the different doses of leptin, was lower than at seven days after treatment (Table I).

**Discussion**

Angiogenesis can be influenced by many factors including hypoxia, growth factors, matrix components and metabolic gradients. Angiogenesis is a biological mechanism of new capillary formation and involves the activation, migration, and proliferation of endothelial cells from preexisting venules (29). The angiogenic activation of endothelial cells probably plays a role in promoting and regulating other biological events, such as inflammation, fibroblast proliferation, extracellular matrix synthesis, and epithelialization in wound healing (30).

Leptin is an endogenous stimulator of both angiogenesis and increased vascular permeability (18, 19). This process is believed to be essential for revascularisation to occur. Leptin is expressed in developing blood vessels and its receptors are found exclusively on endothelial cells (21). The expression of leptin is potentiated in response to ischemia by activated oncogenes and a variety of cytokines (22). Leptin has been demonstrated to mediate angiogenic activity during the proliferative phase of wound healing (23). It plays an important role in survival of skin flaps. Exogenous angiogenic factors applied to the ischemic area of the flap, such as leptin, can promote the survival of flaps.

In the present study, notable differences between the study groups were encountered. Skin flaps that received VEGF and leptin showed improved re-epithelialization and accelerated normal wound healing. In addition, they demonstrated markedly improved tissue survival, particularly following the injection of the higher leptin doses. Differences between the experimental groups were also noted when the tissues were examined under the light microscope. In those animals that received high doses of leptin and these receiving VEGF, the full tissue skin flap contained a greater number of total blood vessels. Such results were not noted with those animals that received either 1 ml saline or leptin with the antileptin blocking factor in each dose category.

Immunohistochemical staining for leptin using the monoclonal antibody anti-rat CD 34 directed against the protein confirmed production of the protein in the healing tissues. The amount of protein noted in each group of specimens could be quantified by the intensity of the antigen-antibody complex deposition and staining. The specimens from the saline, leptin/antileptin-treated groups had significantly less intense staining. Experimental studies (31-38) have demonstrated that VEGF acted directly within ischemic flaps as well as the surrounding tissues to promote the growth and development of new vascular channels and the blood supply through accelerating arterial blood flow. The administration of exogenous VEGF can induce flap angiogenesis, improve the survival of random extensions of axial-pattern skin flaps, and can significantly enhance flap viability.

**Table I. Mean numbers of new vessels in different groups of rats treated with leptin, leptin/antileptin or saline.**

<table>
<thead>
<tr>
<th>Injection</th>
<th>Mean number of new vessels (Microvessel density, MVD)</th>
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<tbody>
<tr>
<td>Control, A1, B1, C1, D1</td>
<td>2</td>
</tr>
<tr>
<td>A</td>
<td>13</td>
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<tr>
<td>B</td>
<td>22</td>
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<tr>
<td>D</td>
<td>29</td>
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<td>E</td>
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A: injection with 100 ngr/ml leptin, B: injection with 250 ngr/ml leptin, C: injection with 500 ngr/ml leptin, D: injection with 1000 ngr/ml leptin, A1: injection with 100 ngr/ml leptin +150 ngr/ml antileptin, B1: injection with 250 ngr/ml leptin +350 ngr/ml antileptin, C1: injection with 500 ngr/ml leptin +750 ngr/ml antileptin, D1: injection with 1000 ngr/ml leptin+1500 ngr/ml antileptin, Control: injection with saline 1 ml.
Our results revealed that treatment of full-thickness skin flaps with exogenously administered leptin was able to accentuate the cellular response augmenting the production of nutrient blood vessels, which, in turn, increased the viability of the vascularized tissue.

Conclusion

This animal model demonstrates that the application of human recombinant leptin may improve survival of ischemic skin flap tissues although the numbers of the animals used in each group was small. The ideal strategy for clinical application of angiogenic growth factors with gene therapy will require further investigation in animal models.

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References