Exogenously-administered Leptin Increases Early Incisional Wound Angiogenesis in an Experimental Animal Model

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Abstract. Background: Leptin is a potent direct angiogenic factor that stimulates endothelial cell migration and activation in vitro, as well as angiogenesis in vivo. In addition, leptin seems to play an important role in clinical angiogenesis by promoting the development of new blood vessels. Objective: To determine the effect of exogenously administered leptin on incisional wound healing in an experimental animal model. Materials and Methods: Sixty-three Sprague-Dawley male mice were used for the study. Full thickness incisional wound was considered as the wound model. The mice were divided into seven groups of nine animals each. Surgical wounds were injected with murine recombinant leptin. Three different leptin doses of 100 pg/ml, 200 pg/ml and 500 pg/ml were used in different animal groups (A, B and C). For each of the three leptin doses used, another animal group was evaluated with a combined injection of leptin and antileptin: 100 pg/ml leptin with 50 pg antileptin, 200 pg/ml leptin with 100 pg antileptin, 500 pg/ml leptin with 250 pg antileptin (A1, B1, and C1), in order to study the inhibitory effect on the leptin factor. Nine mice served as controls. These were injected with 0.3 ml water for injection solution. Mice were sacrificed 3, 7 and 9 days postoperatively. After sacrifice of the animals, the skin was grossly assessed for appearance, colour and texture. Full thickness incisional wounds were dissected for histological examination. A qualitative analysis of angiogenesis in the surgical wound 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 CD34 monoclonal antibody. Results: The most impressive growth of new blood vessels appeared seven and nine days after treatment with the highest leptin doses. There were no significant differences in microvessel density at seven or nine postoperative days among different groups treated with leptin. None of the wounds from the control group, or those from animal groups treated with the combined injection of leptin and antileptin developed any new vessels. Conclusion: Exogenous administration of leptin may increase early tissue angiogenesis in the incisional wound of an experimental animal model.

During the wound healing processes, an abundant blood supply is necessary to meet the enormous local demands of fibroblast proliferation, extracellular matrix synthesis and epithelialisation (1-3). The impairment of the blood supply may be a contributing factor in delayed healing, or nonhealing, of chronic wounds such as diabetic foot ulcers and pressure ulcers, and wounds caused by acute or chronic arterial occlusion (4, 5). Recent advances in the understanding of neovascularisation have made angiogenesis a prime target for therapeutic manipulation in wound healing. Efforts have been made to induce or stimulate new blood vessel formation in order to reduce the unfavourable tissue effects caused by local ischaemia or to enhance tissue repair (6-8). Growth factors, which are now known to play an important role in cell division, migration, differentiation and enzyme production, are also important regulators of wound angiogenesis (9, 10). As a result, an intense interest has been focused on the pharmacological application of angiogenic growth factors in the compromised wound (8, 11-15).

Angiogenesis is a complex and multistage process which is controlled by a variety of factors. Leptin is a potent direct

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Table I. Mean number of new vessels in different groups of mice treated with leptin, leptin + antileptin or WFI.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Days of treatment</th>
<th>Mean number of new blood vessels (microvessel density, MVD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, A1, B1, C1</td>
<td>3, 7, 9</td>
<td>0</td>
</tr>
<tr>
<td>A</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>A</td>
<td>7</td>
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<tr>
<td>A</td>
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<td>B</td>
<td>3</td>
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<td>B</td>
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<td>10</td>
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<tr>
<td>B</td>
<td>9</td>
<td>11</td>
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<tr>
<td>C</td>
<td>3</td>
<td>16</td>
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<tr>
<td>C</td>
<td>7</td>
<td>23</td>
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<tr>
<td>C</td>
<td>9</td>
<td>22</td>
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All injections were of 0.3 ml final volume. A: injection with 100 pg/ml leptin, B: injection with 200 pg/ml leptin, C: injection with 500 pg/ml leptin, A1: injection with 100 pg/ml leptin + 50 pg antileptin, B1: injection with 200 pg/ml leptin + 100 pg antileptin, C1: injection with 500 pg/ml leptin + 250 pg antileptin, Control: injection with WFI.

angiogenic factor that stimulates endothelial cell migration and activation in vitro, as well as 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 participate in clinical angiogenesis as it promotes the formation of new blood vessels (20, 23).

In this study, the effect of leptin on an incisional wound healing model in mice was evaluated. Angiogenesis of healing wounds was examined after administration of exogenous leptin. It was measured at different stages of postoperative tissue re-epithelialization and remodelling using both conventional staining and immunohistochemistry.

Materials and Methods

Animals. Sixty-three Sprague-Dawley male mice, weighing 25-40 g, were used for the study. The animals were housed in individual cages. They were obtained from the Department of Physiology (University of Thrace, Alexandroupolis, Greece) and maintained under a 12-hour-light/12-hour-dark cycle at 22°C until they were 30 weeks of age. They were allowed food and water ad libitum and monitored for body weight. This project was conducted in accordance with the ethical guidelines of the ‘Declaration of Helsinki’. The University’s and the University Ethical Committee approved the study protocol.

Preparation of wound tissues. After induction of general anesthesia with ether, dorsal regions of the mice were shaved and depilated. The animals were placed in a prone position. A 2-cm, longitudinal, linear, full-thickness incision was made on the rat dorsum below the inferior edge of the scapula by a No. 15 scalpel. Haemostasis was obtained by direct pressure using sterile gauze. The wound was sutured with 5/0 nylon sutures in a continuous fashion. All operative procedures were performed under aseptic conditions. Postoperatively, all mice were kept in individual cages. Their body weights were also determined both before and after the end of the experiment.

The mice were divided into seven groups of nine animals each. The wounds were injected with murine recombinant leptin (Cytolab Ltd. / Pepro Tech Asia, Rehovot, Israel) in 0.3 mL phosphate-buffered saline (PBS) per injection. Three different doses of murine recombinant leptin were used: 100 pg/ml (group A), 200 pg/ml (group B) and 500 pg/ml (group C) in 0.3 mL PBS per injection. Three other animal groups were respectively studied using a combined injection 0.3 ml of leptin with antileptin factor (Cytolab Ltd.), (group A1: 100 pg/ml leptin with 50 pg antileptin; group B1: 200 pg/ml leptin with 100 pg antileptin; group C1: 500 pg/ml leptin with 250 pg antileptin) (Table I).

Nine mice served as controls (control group) and were injected with 0.3 ml water for injection (WFI) solution. Mice from each group were sacrificed 3, 7 and 9 days after injection (Table I).

Histological and immunohistochemical evaluation. Full thickness incisional wounds were dissected for histological examination. The specimens were fixed in 10% formalin, stored at 4°C and sectioned longitudinally at a thickness of 2 μm. A qualitative analysis of angiogenesis in the wound was performed following a standard hematoxylin and eosin (HE) stain.

Further analysis was performed by immunohistochemistry. 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 a microwave oven at 600 W for 15 min. The immunohistochemical stain was performed in 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 then they were put in a microwave oven in Trilogy liquid. After cleaning with 3% H₂O₂ and sterilized water, sections were incubated with EnVision-kit (Dako Corporation, Carpenteria, CA, USA) for 25 min. They were incubated with diaminobenzidine (DAB) for 15 min and then washed with PBS for 5 min. The sections were dehydrated through a series of alcohol solutions.

The incubation was performed at a high temperature of 37°C in order to increase the sensitivity of the stain (24). This method improves the immunohistological expression, decreases non-specific staining and allows greater dilution of the initial antibodies (25-27). A control set used histological sections with known positiveness 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 mouse 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 were used to analyze differences between groups.
Comparison was made both between leptin-treated groups, as well as between leptin-treated and leptin/antileptin or control groups. A p-value less than 0.05 was considered statistically significant.

Results

From gross examination, the incisional wound surfaces healed without any complications in both experimental and control groups at both 7 and 9 days, postoperatively.

It was found that group C animal wounds, treated with 500 pg/ml of leptin, had the greatest number of new vessels in comparison to the other six groups. The second best performance in terms of angiogenesis was that of the group B wounds treated with 200 pg/ml of leptin.

The greatest formation of new vessels for all 3 doses of leptin was apparent seven days after injection. There was growth of inflammatory granulous tissue, hyperplasia of squamous epithelium and an increase in fattening and irregular deposition of the 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. This comparison is shown in Figure 2.

None of the nine wounds from the control group or the twenty-seven injected with the combination of leptin and antileptin demonstrated any formation of new blood vessels. There was growth of inflammatory granulous tissue, with inflammatory cells of mixed type, fibrinoblasts and fibrins of collagen instead (Table I). Most of the wounds in the third postoperative day for all three different doses of injected leptin had growth of inflammatory granulous tissue, fibrinoblastocells and fibrins of collagen, as well as initial formation of new vessels, particularly at the wound edges. There was broad development of fibroblasts focused toward the center with no signs of necrosis (Table I). Nine days after treatment, there was marked hyperplasia of squamous epithelium, increase, fattening and irregular disposition of the collagen fibers. There was also broad appearance of fibroblasts focused toward the center of the wound with no signs of necrosis. The mean number of new blood vessels developed was less than that at seven days after treatment (Table I).

Discussion

Angiogenesis is the biological mechanism of new capillary formation. It involves the activation, migration and proliferation of endothelial cells from preexisting venules. It can be influenced by factors such as hypoxia, matrix components, metabolic gradients and growth factors (1, 2).

Growth factors driving re-epithelialization are crucial to the wound-healing process. Important roles for this process have been elucidated for keratinocyte growth factor (KGF), epidermal growth factor (EGF) and transforming growth factor (TGF)-α, which have been shown to stimulate re-epithelialization in animal models or to be absent in models of impaired re-epithelialization. In line with these observations, keratinocytes of the hyperproliferative epithelium at the wound edge and endothelial cells are known to express the KGF- or EGF-receptor, respectively (28).

Repertinger et al. (29) demonstrated that epidermal growth factor receptor (EGF-R) regulates multiple facets of cutaneous wound healing, including inflammation, wound contraction, proliferation, migration and angiogenesis.

Galiano et al. (30) demonstrated that pharmacological vascular endothelial growth factor (VEGF) therapy in diabetics enhances neovascularization with a clinically significant effect. The mechanism for this effect is through the stimulation of local angiogenesis, enhanced expression of growth factors including PDGF and FGF-2, and systemic mobilization of bone marrow-derived stem cells. This combination of effects is likely responsible for the increased perfusion, improved peripheral neuropathy and enhanced collateral formation. Because VEGF is uniquely able to enhance local angiogenesis and mobilize endothelial progenitors into the circulation, VEGF therapy may be exploited to promote tissue repair in a wide variety of acute and chronic injuries, particularly in conditions such as diabetes or aging.

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 epithelialisation in wound healing.

Leptin is an endogenous stimulator of both angiogenesis and increased vascular permeability (18, 19). This process is believed to be essential for neovascularization to occur. Leptin is expressed in developing blood vessels and its receptors are found exclusively on endothelial cells (21). The expression of leptin is believed to be potentiated in response to ischaemia 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). The beneficial effect of leptin on wound repair is due to a direct mitogenic action of leptin on keratinocytes located at the wound margins (31).

In the present study, notable differences between the study groups were encountered (Table I). Blood flow measurements varied significantly in various groups of animals. More importantly, there was improved re-epithelialization of incisional wounds in mice and accelerated normal wound healing after leptin administration. Wounds that received leptin had markedly improved tissue survival, especially for the higher doses and at 7 or 9 days after surgery.

Differences between the experimental groups were also noted when the tissues were examined under light microscopy. In those animals that received leptin, the subcutaneous tissue supplying the wound contained a greater total number of
blood vessels. Similar results were not noted with those animals that received either WFI or leptin with the blocking factor antileptin in every dose used, even at 7 or 9 days postoperatively. This may be attributed to the manipulation of the wound without the addition of beneficial gene therapy.

Immunohistochemical staining confirmed the 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 WFI - leptin/antileptin treated groups had significantly less intense staining.

The results demonstrated that treatment of the wounds with recombinant leptin can accentuate the cellular response producing increased amounts of leptin as a means of augmenting the production of nutrient blood vessels which, in turn, increases the viability of the vascularized tissue. These experimental findings are in accordance with previous studies. Shafer et al. (4) suggested that leptin modulates vascular remodeling in vivo and that elevated leptin levels dramatically promote lesion growth after experimental vascular injury in the mouse. Frank et al. (31) on the other hand, demonstrated that leptin-triggered epithelial processes are crucially involved in the wound-healing deficiencies observed in ob/ob mice.

They identified leptin as a potent mediator of keratinocyte proliferation during wound healing in vivo. Their study provided evidence for beneficial effects of topically applied leptin for normal wound-healing situations.

**Conclusion**

This animal incisional wound healing model demonstrates that the application of exogenous leptin could improve angiogenesis in wound tissue. It provides strong evidence of an angiomodulatory effect that leptin may have in complicated wound treatment.

**References**


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