Heparin administration expands flap survival area possibly by increasing the concentration of hepatocyte growth factor

Takeshi Kan[1], Hirotaka Asato[1], Norio Fukuda[2,] Goro Takada[1], Shoichi Sasaki[1]

[1] Department of Plastic and Reconstructive Surgery, Dokkyo Medical University School of Medicine, Tochigi 321-0293, Japan

[2] Department of Dentistry, Oral and Maxillofacial, Plastic and Reconstructive Surgery, Yamagata University School of Medicine, Yamagata 990-9585 Japan

Correspondence: Takeshi Kan, MD[1]

Tel: +81-282-87-2485

Fax: +81-282-86-1806

E-mail: tkan@dokkyomed.ac.jp

Abstract

Hepatocyte growth factor (HGF) has a strong angiogenic effect on various organs. Rapid administration of intravenous heparin increases human plasma HGF concentration. Angiogenesis has a positive effect on flap survival, and the angiogenic effect of administered heparin is expected to expand the flap survival area. Previous reports stated that heparin administration could expand the flap survival area, but they did not focus on the angiogenesis effect of heparin but instead its anti-coagulation and anti-inflammatory effects. Therefore, we studied the effects of heparin administration on flap survival in an animal model and expected its angiogenic effect to expand the flap survival area. Twenty male Wistar rats (8-9 weeks old) were randomly divided into two groups of ten: heparin and control groups. Distally based McFarlane flaps were raised in all rats. In the heparin group, 300 U/kg of heparin was administered daily, whereas, in the control group, saline was administered daily. We compared the flap survival area and the number of new blood vessels on postoperative day 7. The mean flap survival areas were significantly higher in the heparin group (57.9±5.3%) than in the control group (47.3±5.9%) ($P<0.01$). The number of CD31-positive cells in ten high power field images was significantly higher in the heparin group (60.3±7.3 cells) than in the control (43.7±4.8 cells) ($P<0.01$). In our murine model, heparin administration positively affected flap survival and angiogenesis. We consider that the increased serum HGF concentration via heparin administration is responsible for this result.

Keywords: Flap survival area, hepatocyte growth factor, angiogenesis, heparin administration

Introduction

Flap surgery is an essential surgical technique when reconstructing the skin in a defected area. Blood supply of a flap from the underlying tissue is severed after flap elevation, as blood supply relies on vascular connections to the skin pedicle. A flap with a ratio of 1:2 (width: length) has enough blood supply to remain viable, while a flap with a ratio beyond 1:2 tends to develop necrosis from ischemia. Establishing adequate blood supply from surrounding tissue is critical for flap survival to avoid ischemia and necrosis.

Hepatocyte growth factor (HGF) was identified as a potent hepatocytic mitogen by Nakamura et al. [1]. Additional experiments revealed that HGF affects tissue regeneration by evoking multiple cellular responses, including mitogenicity, motility, and morphogenesis [2-5]. The angiogenic activities of HGF in ischemic and injured areas are more robust than those of vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF). HGF promotes endothelial cell proliferation and vascular smooth muscle cell migration [6]. HGF is challenging to use in clinical practice because of its short half-life and higher molecular size; therefore, there are currently no available treatments that employ HGF to its potential.

Okada et al. [7] and Salbach et al. [8] reported that rapid heparin administration transiently elevates serum HGF concentration in a dose-dependent manner. It was proposed that heparin induces HGF to migrate and adhere to the extracellular matrix of the vessel wall and subsequently increases its concentration. Further, heparin activates HGF in HGF target cells and promotes

fibroblast production. As such, intravenous heparin administration remains an angiogenic therapy for coronary and peripheral artery diseases [9]. Previous reports have stated that heparin administration could expand the flap survival area; however, they did not focus on the angiogenesis effect of heparin but instead focused on its anti-coagulative and anti-inflammatory effects [10-12].

During flap surgery, increased HGF concentration from rapid intravenous heparin administration was expected to cause angiogenesis of the flap and its surrounding tissues, expanding the flap survival area. We report the effects of heparin administration on flap survival and its relationship to angiogenesis in a murine model.

Materials and Methods

Twenty male Wistar rats (Wistar/ST rat, age: 8-9 weeks, weight: 305-350 g; SLC,Shizuoka Japan) were evenly divided into two groups by random allocation to either the control or the heparin group. All rats received unrestricted access to food and water. We adhered to the guidelines of Dokkyo Medical University for the care and use of laboratory animals.

A distally based McFarlane flap (full thickness; random pattern skin flap including panniculus carnosus; 3×9 cm in size) was elevated in all rats and then returned to the original site and fixed with 4-0 Nylon suture [13]. All surgeries were performed under general anesthesia with isoflurane inhalation combined with local anesthesia using lidocaine. Treatments of either saline (control group) or 300 U/kg heparin (heparin group) were rapidly administered postoperatively. We

administered 0.1 to 0.15 ml of saline to the control group to ensure administration of the same amount of heparin per kg of body weight to the heparin group. Intravenous injection was performed by inserting a 30 G needle with a 1 ml syringe into an exposed internal jugular vein or femoral vein at every injection. We did not use an intravenous catheter because extra heparin would be needed to prevent catheter obstruction and the extra heparin could have affected the result. Saline or heparin was administered daily until postoperative day 7 when we harvested samples to compare the flap survival area and the number of new blood vessels.

Photographs of the back of the rats (postoperative day 7) were obtained to quantify the flap survival area using AreaQ™ image analysis software (S-Tech Corp., Chiba, Japan). Using this program, the length of the image was converted to the actual length and the surface area was calculated. The regions of survival and necrosis were clearly demarcated in every flap. The survival area appeared pink-white, tender and normal in its texture. In contrast, the necrotic area was black, hardened and covered with crust. The survival area was determined by subtracting the necrotic area from the total flap surface area. The mean percentage of flap survival area was compared between the groups.

The flap tissue (10×10mm) was harvested 3 cm from the flap base on postoperative day 7 and fixed with 10% formaldehyde. Formaldehyde-fixed tissues were transferred into a paraffin-embedded block. To visualize the new blood vessels, vertical consecutive sections (5 μm) were performed and stained with CD31 immunostaining. The number of CD31-positive cells was counted in ten randomly chosen high- powered field

images of each sample. We compared the mean number of CD31-positive cells between groups.

Two rats from each group were randomly selected and then prepared for the angiography that was executed on postoperative day 7. Immediately before the angiography, a thoracotomy was performed to inject 60 mL of lactated Ringer's solution and the radiopaque mixture via the left ventricle replacing intravascular blood. The radiopaque mixture consisted of 100 g lead oxide, 10 g powdered milk, and 500 mL water (40 ℃) [14]. After injection, the skin of each rat's back was harvested immediately, and a skin angiography was performed at 23 kV for 0.17 s with MAMMOMAT Inspiration (SIEMENS AG, Erlangen, Germany).

Statistical significance was assessed using a non-parametric Mann-Whitney *U* test and 95% confidence intervals. The difference was considered significant if the P-value was <0.05.

Results

The border between the surviving and necrotic flap areas was observable with the naked eye. On postoperative day 1-2, the tip of the flap turned dark purple. This dark purple area scabbed by postoperative day 3-4. On a postoperative day 5-6, any necrotic areas became evident. No rats died during the experiment, and we did not observe any infection, wound dehiscence, or hematoma at the surgical site.

The mean survival area of the flap was 47.3±5.9% in the control group and 57.9±5.3% in the heparin group. The flap survival area was more significant in the heparin group than in the control

group ($P<0.01$) (Figures 1 and 2). The number of CD31-positive cells in 10 high power fields was 43.7±4.8 cells in the control group and 60.3±7.3 cells in the heparin group ($P<0.01$) (Figure 3 and Figure 4). Since the number of CD31-positive cells correlated with the number of new blood vessels, we concluded that the number of new vessels was higher in the heparin group than in the control group.

In angiography, the vascular network was evenly distributed on the skin of unoperated rat's back. The vascular network was cut by surgery, but angiography on postoperative day 7 showed that the new vascular network was being reconstructed. Upon angiography analysis, there was more active angiogenesis in the heparin group's flap margin than in the control group's flap margin. In the flap's suture-fixed area, a newly established blood plexus was observed in the heparin group (Figure 5).

Discussion

In this study, heparin administration after flap surgery promoted flap angiogenesis and expanded the flap survival area. Previous reports have stated that heparin administration could expand the flap survival area; however, they did not focus on the angiogenesis effect of heparin but instead focused on the anti-coagulative and anti-inflammatory effects [10-12].

Multiple methods have been tested to improve flap perfusion, including dilating vessels of flaps with a substance, applying mechanical stimuli, and improving the metabolism of flap tissue [15-

17]. Recently, gene therapy, including growth hormone administration (e.g., bFGF, VEGF, HGF, and platelet-derived growth factor) and stem cell transplantation (e.g., bone marrow-derived and adipose-derived stem cells) have attracted the attention of many researchers [18-24]. Although gene therapies show favorable results in animal experiments, there are currently no standardized gene therapies in clinical practice. We attribute this outcome to the complexity, safety, and high cost of those methods.

Heparin seems to contribute to angiogenesis in ischemic areas and damaged organs by increasing serum HGF concentration without a direct effect on angiogenesis. It has been proposed that HGF plays an essential role in the proliferation and functional improvement of endothelial cells in damaged organs, more potently promoting angiogenesis than bFGF and VEGF [2, 6]. HGF is produced by hepatic vascular endothelial cells of the liver, the lungs, and the damaged organs. It binds loosely to the vascular extracellular matrix and is stored as intrinsic HGF. Heparin may have a high affinity for HGF, and heparin administration may increase serum HGF concentration by dissociating HGF from the extracellular matrix of endothelial cells into the circulation in a dose-dependent manner. Increasing serum heparin concentration by rapid administration is essential to release HGF from the extracellular matrix of endothelial cells [7, 8].

This study showed that the flap survival area was more significant in the heparin group than in the control group. Further, the number of CD31-positive cells in the flaps increased in the heparin group. Our results suggest that heparin administration induced angiogenesis in the flaps. In the

angiography, more active angiogenesis was observed in the marginal area of the flap in the heparin group than in that of the control group, and the newly established blood plexus was observed in the suture-fixed area of the flap in the heparin group.

Once HGF is released into the serum, it exerts many biological effects through c-Met receptors of the target organs, including damaged organs. The expression of c-Met is upregulated in ischemic conditions [5], whereas HGF concentration decreases in these conditions [25]. Decreased HGF concentration may cause delays in repair and proliferation of endothelial cells, albeit increased c-Met expression by decreased circulation. In such a condition, supplementing HGF seems possible. Heparin administration, which indirectly increases HGF levels, might contribute to the expansion of the flap survival area by promoting angiogenesis and endothelial regeneration and improving the endothelial function in the flaps and suture-fixed areas. Heparin also has an anti-inflammatory effect by inhibiting eosinophil and neutrophil infiltration [10-12]. The synergistic anti-coagulation and anti-inflammatory effects, which occur directly from administered heparin and the subsequent elevated HGF concentration, may result in a broadened flap survival area. Specifically, we suggest that elevated serum HGF concentration has a significant role in promoting angiogenesis in flaps.

In this research, we did not evaluate serum HGF increase after heparin administration in the rats and could not directly describe the relationship between serum HGF and flap survival area. Matsumori et al stated that serum HGF concentration in rats increased after administration of 30, 100 and 300 IU/kg heparin and concluded that heparin injection of 300 IU/kg showed the highest

increase in serum HGF. Following that research, we determined the dose of heparin administration as 300 IU/kg [3]. Because intrinsic HGF might have reached a maximum because of daily heparin administration and no elevation of HGF concentration, to determine whether HGF concentration had a significant role in promoting angiogenesis, continuous measurement of HGF concentration would have been insightful. Although the half-life of HGF is a few minutes and HGF concentration returns to normal levels within 4 hours of heparin administration [8, 9], the effect of elevated serum HGF concentration on angiogenesis in the flaps has been confirmed in this study. However, we did not evaluate potential adverse effects, such as bleeding. Supplemental studies to evaluate serum HGF changes after heparin administration and to determine the appropriate dose of heparin should be conducted in the future. In addition, we evaluated pathological specimen only on postoperative day 7 and did not evaluate the chronological changes in this research. Comparison of the chronological changes in flap survival area and pathological specimen is needed in the future as well.

In this experiment, rather than using exogenous HGF, which is unstable due to its high molecular weight, we used intrinsic HGF and avoided these hurdles. Also, the rapid heparin administration we used was inexpensive and convenient. Heparin administration increased the number of blood vessels in the flap and significantly expanded the flap survival area. We suggest that elevated HGF concentration has a significant role in promoting angiogenesis in flaps. This method might be adapted to not only flap surgery, but also other surgeries related to angiogenesis, including skin

graft and free flap surgeries.

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Declaration of Interest Statement

No potential conflicts of interest were reported by the authors. The authors alone are responsible for this paper's content.

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