Effects of inhibition of lymphangiogenesis by the vascular endothelial growth factor receptor 3 (VEGFR-3) inhibitor, MAZ51 on full thickness wounds in mice

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Abstract

We herein used MAZ51 to inhibit lymphangiogenesis and aimed to clarify the effect of inhibition of lymphangiogenesis on wound healing. BALB/c male mice were divided into two groups: the control group which was injected the dimethyl sulfoxide (DMSO), the experiment group was injected MAZ51 in the DMSO. All wounds were observed for 15 days and the wound areas were measured. Tissue samples were harvested on day 3, 7, 9, 11, 13 and 15, and subjected to immunostaining of blood vessels and lymphatic vessels. There are no significant differences between two groups in the wound area, the number of blood vessels and lymphatic vessels. The number of blood vessels peaked on day 7 in both groups, while the number of lymphatic vessels peaked on 13 or 15 in both groups. This result revealed delayed lymphangiogenesis in comparison with previous studies. The wound healing process in the control and experiment groups were similar, but both groups seemed delayed lymphangiogenesis comparing with previous studies. Injections of MAZ51 or/and DMSO did not affect angiogenesis, while they may affect lymphangiogenesis.

Introduction

Wound healing is a primitive but complex process that restore cellular structures and skin layers. Cutaneous wound healing immediately begins after injury and is divided into three phases: inflammation, proliferation, and remodeling.1 Lymphatic vessel growth or lymphangiogenesis is a part of restoring process on wound healing. However, little is known about the role of lymphatic vessels on wound healing process in comparison to the blood vessels.2

Tissue lymphangiogenesis typically occurs at sites of inflammation induced by Vascular endothelial growth factor (VEGF)-C produced by infiltrating macrophages.3-5 In our previous study,6 this process contributes to reduce wound exudate and tissue edema. However, it is not unclear whether other immune cell types contribute lymphangiogenesis in inflamed tissue.

VEGF-C is lymphangiogenic factors that bind to and activate VEGF receptor 3 (VEGFR-3) which is a tyrosine kinase receptor expressed on lymphatic endothelial cells in the adult. MAZ51 (4-dimethylamino-naphthalene-1-carbaldehyde), VEGF-3 Kinase Inhibitor, has been described by Kirkin et al.7 to block VEGF-C-induced VEGFR-3 kinase activity. MAZ51 inhibits phosphorylation of VEGF-3 induced by VEGF-C and impairs lymphangiogenesis. In vitro experiment, MAZ51 blocked the proliferation of VEGFR-3-expressing human endothelial cells, while in vivo experiment, it significantly inhibits the growth of rat mammary carcinomas.8 However, it is still unknown whether MAZ51 inhibit lymphangiogenesis on wound healing process. We herein used the MAZ51 to inhibit lymphangiogenesis and aimed to clarify the effect of inhibition of lymphangiogenesis on wound healing process.

Materials and Methods

The experimental protocol and animal care procedures were in accordance with the Guidelines for the Care and Use of Laboratory Animals of Kanazawa University, Japan. This experiment was approved by the Committee on Animal Experimentation of Kanazawa University (AP-091215).

Animals

Fifty-four BALB/cCrSlc male mice (Sankyo Lab Service Corporation, Inc., Toyama, Japan) weighing 19.4-25.1 g were used. They were caged individually in an air-conditioned room at a temperature of 25.0±2.0°C, and the lights were kept on from 08:45 to 20:45 h. Water and pelleted food were given freely.

Inhibition of lymphangiogenesis

MAZ51 is a kinase inhibitor of VEGFR 3. Mice in the experimental group were subcutaneously injected daily with MAZ51 (10 mg/kg body weight) (Merck KGaA, Darmstadt, Germany) in dimethyl sulfoxide (DMSO) (Wako Pure chemical Industries, Ltd., Osaka, Japan) based on the previous study.9 Mice in the control group were subcutaneously injected daily with DMSO at the same dose of the control group.

Wounding

The day before wounding, the mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (0.05...
mg/g weight) and the dorsum was shaved. Next day, two circular full-thickness skin wounds (4 mm in diameter), including the panniculus carnosus muscle on both sides of the dorsum of the mouse, were made with a sterile disposable biopsy punch (Kai Industries Co, Gifu, Japan). The day wounding was designated as day 0. Wounds were covered with a hydrocolloid dressing (Tegaderm, 3M Health Care, Tokyo, Japan) to maintain a moist environment, and then mice were wrapped twice with adhesive bandages (mesh pore tape, Nichiban, Tokyo, Japan). The bandages and hydrocolloid dressings were changed every day and wound healing was observed daily until day 15 after wounding.

**Macroscopic observations**

The wound healing process was observed every day after wounding. After anesthetize with pentobarbital sodium, the wound edges were traced on polypropylene sheets, and photographs were taken at each wound. The traces on the sheets were captured with a scanner and transferred to a personal computer using Adobe Photoshop Elements 7.0 (Adobe System Inc., Tokyo, Japan), and the area of each wound was calculated using the image analysis software Scion Image Beta 4.02 (Scion Corporation, Frederick, Maryland, USA). The wound areas were expressed as the ratios using the following formula: the area of each observation day/the area on day 0.

**Tissue processing and staining**

The mice were euthanized via the IP injection of a large dose of pentobarbital sodium (0.5 mg/g weight) on day 3, 7, 9, 11, 13 and 15. The wounds and the surrounding intact skin were harvested, stapled onto transparent plastic sheets to prevent the specimens contracting excessively, and fixed in Zinc fixative solution (BD Pharmingen, CA) for 24 h. The specimens were then dehydrated in an alcohol series, cleaned in xylene, and embedded in paraffin to prepare 5-µm serial sections. These sections were stained with hematoxylin-eosin (H-E) or immunohistologically stained with anti-lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) antibody (RELIATech GmbH, Germany) to detect lymphatic endothelial cells, anti-CD31 antibody (BD Pharmingen, CA) to detect blood vessels.

After deparaffinization and rehydration, the sections were washed with phosphate-buffered saline (PBS), and incubated with anti-mouse LYVE-1 antibody or anti-CD31 antibody at 4°C overnight or for one hour at room temperature. Light hematoxylin countercstaining was applied for 1 minute to visualize the cell nuclei. Negative control sections were obtained by omitting each primary antibody.

**Microscopic examination**

In order to evaluate the lymphatic vessels, we counted the number of lymph vessels in the granulation tissue during examinations performed with a light microscope at a magnification of 20x. The number of lymph vessels in the granulation tissue (number/mm²) was calculated as follows: the number of lymphatic vessels detected by LYVE-1 in the entire granulation tissue/the entire area of granulation tissue. The number of blood vessels detected by CD31 was calculated as follows: the number of blood vessels at three areas in the granulation tissue of one specimen/ the sum total area of the three areas.

**Statistical analysis**

Data were expressed as mean ± SD values and were compared using t-test by JMP ® 8.0.1 (SAS, USA) (ANOVA, multiple comparisons Tukey-Kramer). The differences were considered significant at P<0.05.

**Results**

**Effect on wound healing process**

Wound healing processes in the control and experiment group were similar according to macroscopic observation (Figure 1A). New epithelium was observed at the wound edge around day 4 or 5 after wounding, and all wounds had completed re-epithelialization between days 10 and 15. Wound areas in the control and experiment groups increased after wounding, and peaked on day 4 at 1.5±0.3 and 1.6±0.3, respectively compared with the initial wound area, and decreased gradually to 0.2±0.1 and 0.2±0.1, respectively on day 15 (Figure 1B). There were no significant differences between both groups during experimental follow-up.

**Effect on blood vessels**

We used the anti-CD31 antibody to detect blood vessels in the granulation tissue (Figure 2). The number of blood vessels peaked on day 7 in both groups, and blood vessels were observed in the entire wounds. After day 7, they decreased gradually until day 15. There were no significant differences between both groups. The number of blood vessels in both groups were higher than that in normal skin.

**Effect on lymphatic vessels**

We used the anti-LYVE-1 antibody to detect lymphatic vessels in the granulation tissue (Figure 3). The number of lymphatic vessels peaked on day 13 in the experiment group, while it peaked on day 15 in the control group. The number of lymphatic vessels in both groups were less than that in normal skin until day 15. There were no significant differences between both groups during experimental follow-up although the number of lymphatic vessels in the experimental group seemed to be lower than that of the control group on day 9, 11, and 15.

**Discussion**

In this study, the wound healing process in the experiment group was similar with that in the control group although the experiment group was daily received MAZ51 to inhibit lymphangiogenesis. However, comparing with the ratio of wound area in this study with that in our previous studies, both groups seemed delayed wound healing in

![Figure 1. Macroscopic examination of wound healing. A) Representative wound images for the control and experiment group. Bar: 5 mm, n=17 on each group. B) The ratio of wound area. No significant differences (N.S.) were observed during experimental follow-up. P<0.05, t-test.](image-url)
this study. The wound ratio in the control group in our previous studies12-14 which was covered with a hydrocolloid dressing were 0.1, 0.12, and 0.01 on day 15, while that in this study was 0.2±0.1 in both groups on day 15. Angiogenesis detected by anti-CD31 antibody immunostaining peaked on day 7 in both groups and gradually decreased at the similar levels of normal skin. This result was consistent with the previous studies.12,13 Thereby, we focused on lymphangiogenesis in this study.

Concerning with lymphangiogenesis in this study, the lymphatic vessels appeared in the granulation tissue on day 7, their numbers peaked on day 15 in the control group and on day 13 in the experiment group, and these numbers of lymphatic vessels were less than that in normal skin. Paavones et al.15 reported that the number of lymphatic vessels peaked on day 5 in punch biopsy wounds in pigs. Shimamura et al.12 reported that, in full thickness wound in mice, lymphatic vessels appeared on day 7 and its number peaked on day 11, which number was greater than normal skin. Therefore, it suggested that lymphangiogenesis in both groups was delayed and inhibited in this study. It may affect the daily injection of DMSO or/and MAZ51. Comparing the experiment group with the control group, injection of MAZ51 did not show the inhibition of lymphangiogenesis in the experiment group clearly. VEGF-C/VEGFR-3 signaling pathway is essential for lymphangiogenesis, so we attempted to inhibit this pathway using MAZ51. However, Cao16 reported that the platelet-derived growth factor (PDGF) family are potent angiogenic factors, particularly, PDGF-BB are able to induce lymphangiogenesis and VEGFR-3 antagonists are unable to inhibit PDGF-BB-induced lymphangiogenesis in adult animal. In this study, although we did not examine the relationship between lymphangiogenesis and PDGF-BB, the little effect of MAZ51 on inhibition of lymphangiogenesis might be attributed to the independent signaling pathway of PDGF-BB from VEGF-C/VEGFR-3 signaling pathway.

Conclusions

The wound healing process in the experiment group was similar with that in the control group although the experiment group was daily received MAZ51 to inhibit lymphangiogenesis. However, the number of lymphatic vessels peaked on 13 or 15 in both groups. This result revealed delayed lymphangiogenesis. Injections of MAZ51 or/and DMSO did not affect angiogenesis, while they may affect lymphangiogenesis.

Limitation of this study

We used MAZ51 to inhibit lymphangiogenesis, but it is unclear the effect of MAZ51 on inhibition of lymphangiogenesis on wound healing process. We did not examine the effect of PDGF-BB on lymphangiogenesis. We will investigate these issues in future.

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Figure 2. Angiogenesis detected by anti-CD31 antibody immunostaining. A) The number of blood vessels in the granulation tissue. No significant differences were observed during experimental follow-up. P<0.05, t-test. n=6 on each group. B) Representative histological images on day 7. Numerous new blood vessels were observed in the granulation tissue. Dotted lines: boundary between wounds and wound edges.

Figure 3. Lymphangiogenesis detected by anti-LYVE-1 antibody immunostaining. A) The number of lymphatic vessels in the granulation tissue. No significant differences were observed during experimental follow-up. P<0.05, t-test. n=6 on each group. B) Representative histological images on day 15. New lymphatic vessels revealed by arrows were observed in the granulation tissue. Dotted lines: boundary between wounds and wound edges.
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