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メタデータ	言語: eng 出版者: 公開日: 2017-10-03 キーワード (Ja): キーワード (En): 作成者: メールアドレス: 所属:
URL	http://hdl.handle.net/2297/19135

Relationship between lymphangiogenesis and exudates during wound healing process of mouse skin full thickness wound

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Short running title:

Lymphangiogenesis and exudates during wound healing

Key words:

exudate, new lymphatic vessels, new blood vessels, cutaneous wound healing, mouse, Indian ink

ABSTRACT

We considered the relationship among exudate, wound area, angiogenesis, lymphangiogenesis, and reepithelization during wound healing. Full thickness wounds were made on the dorsum of mice. The weight of exudate absorbed into the dressing as well as the wound area was determined daily. Sections of the wounds were stained with anti LYVE-1 and CD31 antibodies. Indian ink was injected into the wound for observing the movement of the exudate on days 3, 5, and 7 after wounding. New epithelium completely covered the wound on day 11. The quantity of exudate peaked on day 1, and was rapidly reduced until it was not detected on day 11. Most Indian ink injected into the wound was retained in the wound and did not flow into the surrounding tissue. New blood vessels showed uniform distribution in granulation tissue on day 5. New lymphatics appeared in the granulation tissue approximately 2 days later than blood vessels and were distributed at the center of the granulation tissue on day 11, and thus reduction of exudate from the wound seems related to blood vessels, not lymphatics. However, increasing lymphatics may play a role in the late phase of the wound healing process.

INTRODUCTION

Healing of cutaneous full-thickness wounds has been arbitrarily divided into three phases: 1. inflammation, 2. re-epithelialization and granulation tissue formation, and 3. matrix formation and remodeling.¹⁻³ The three phases of wound repair, however, are not mutually exclusive but rather overlap in time. In the inflammation phase, severe tissue injury causes blood vessel disruption with concomitant extravasation of blood constituents. Blood coagulation and platelet aggregation generates a fibrin-rich clot that plugs severed vessels and fills any discontinuity in the wounded tissue. Several protein-rich plasma and inflammatory cells leave the vessels to the wounded tissue to make a massive exudate. The exudate overflows from the wound surface. In granulation tissue formation, new stroma called granulation tissue begins to form approximately 4 days after injury, and this consists of numerous new capillaries and fibroblasts, loose connective tissue and inflammatory cells. The capillaries provide nutrition and oxygen to rapidly proliferating and migrating epidermal keratinocytes, fibroblasts and inflammatory cells. Lymphatic vessel regeneration also occurs a few days^{4, 5} or one week^{6, 7} after blood vascular regeneration. In the remodeling phase, fibroblasts transform into myofibroblasts that contract the wound, epidermal cells differentiate to reestablish the permeability barrier, and a large amount of extracellular matrix is deposited. Vascular endothelial cells undergo apoptosis⁸ and the number of blood vessels decrease, followed by a formation of a rather acellular scar. Non-lymphatic vessels stained with anti VEGF-3 antibody are observed.⁴

A large amount of exudate is observed during the healing of cutaneous full-thickness wounds.⁹⁻¹¹ Since the exudate produced by the permeability of the blood vessels stimulated by injury includes many inflammatory cells, matrix components, biological factors, proteases, and cytokines,¹²⁻¹⁵ it plays an important role for wound healing. Under the benefit of moisture, wound healing is improved, and in dry conditions, little exudate

slows wound healing.^{16, 17} Moreover, too much wound surface moisture also delays the wound healing.¹⁸ The goal of effective wound management is to remove excess moisture, debris, and chemicals from the wound surface, while maintaining the ideal moisture balance to allow cell migration and ultimately wound healing.¹⁹

The circulation system in the integumentary system consists of blood and lymphatic vessels as well as other organs. The lymphatics can carry proteins and large particulate matter away from the tissue spaces, neither of which can be removed by absorption directly into the blood capillaries. The lymphatic system takes part in the immune response, and metastasis of malignant tumors.^{20, 21} It is well known that during inflammation, lymphatic flow is increased and helps drain the edema fluid from the extravascular space, and localized lymphedema occurs with impaired lymphatic drainage due to inflammatory or neoplastic obstruction.²² Thus, the impaired lymphatics may cause delayed healing of cutaneous wounds.²³

In this study, we considered the relationship among exudate from a wound surface, wound area, angiogenesis, lymphangiogenesis, and reepithelization during the wound healing process, and moreover whether the exudate in the wound leaked from the wound surface or was absorbed into the lymphatics surrounding the wound.

Materials and Methods

Materials

Animals

Forty-five C57BL/6CrSlc male mice aged 8 weeks and weighing 21.9-28.0 g were used. They were caged individually in an air-conditioned room at $25.0 \pm 2.0^{\circ}\text{C}$ with light from 08.45 to 20.45 hours. Water and laboratory chow were given ad libitum.

The experimental protocol and animal care were in accordance with the Guidelines for the Care and Use of Laboratory Animals of Kanazawa University, Japan.

Methods

Wounding and calculating of exudate and wound areas

Mice were anesthetized with i.p. injection of pentobarbital sodium (0.05 mg/g weight). Two circular (4 mm in diameter) full-thickness skin wounds including the panniculus on both sides of the dorsum of the mouse were made with a sterile disposable biopsy. The day when wounds were made was designated as day 0.

Wounds were covered with hydrocolloid dressing (Tegasorb; 3M Health Care Ltd., Tokyo, Japan) to maintain a moist environment and to absorb exudate leaked from the surface of the wound. Mice were wrapped with sticky bandage (Meshupore®, Nichiban Co., Ltd., Tokyo, Japan) so the Tegasorb did not slip out of position. Hydrocolloid dressing was cut into squares (18 mm x 18 mm) and the weight of each was measured before the wound was covered. The following day, the hydrocolloid dressing was changed. The dressing and the absorbed exudate was weighed to calculate the weight of the exudate (Fig. 1). The weight of exudate on day 0 after wounding was determined as the weight of the dressing with exudate on day 1 minus the weight of the dressing on day 0. From day 0 after wounding to day 15, this process was repeated every morning.

Additionally, the process of wound healing was observed from days 0 to 15 after wounding. Wounded edges were traced on polypropylene sheets and photographs were

taken every day. The traces on the sheets were captured with a scanner onto a personal computer by Adobe Photoshop Element 2.0 (Adobe Systems Inc., USA), and the areas of wounds were calculated using image analysis software Scion Image Beta 4.02 (Scion Corporation, Maryland, USA).

Injection of Indian ink into wounds

On days 3, 5, and 7, 0.02 mL Indian ink was injected into both sides of the wounds with a 26G needle and the sites were covered with transparent dressing for 2 hours. For controls, 0.02 mL Indian ink was injected into intact back skin. After this, the tissues were harvested.

Histological procedure

The mice were euthanized by massive pentobarbital sodium i.p. injection on days 1, 3, 5, 7, 9, 11, 13, and 15 after wounding. The wounds and the surrounding intact skin measuring 15 mm x 15 mm square were harvested, stapled onto transparent plastic sheets to prevent over-contraction of the samples, and fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 12h for paraffin sections, or in PLP fixation for 12 h for cryosections, or cryoembedded in OCT compound in liquid nitrogen without chemical fixation for cryosection, or fixed in 2% glutaraldehyde in 0.1 M phosphate buffer, pH7.4, for transmission electron microscopy.

Some samples embedded in paraffin were sectioned perpendicular to the wound surface and the others were sectioned horizontal to the wound surface. All samples embedded in OCT compound were sectioned perpendicular to the wound surface with a cryomicrotome.

Sections of 5 μ m in thickness were stained with hematoxylin and eosin, and immunostained with anti CD31 antibody for detecting blood vessels or anti LYVE-1 antibody for detecting lymphatic vessels, or with both antibodies.

Immunostaining procedure

Individual antibodies were tested at various dilutions to obtain the optimal working concentrations for use in an immunostaining procedure. Some paraffin sections were first deparaffined, dehydrated, and washed in 0.01 M PBS. They were retrieved by heating in citrate buffer solution, pH 6 at 95°C for 40 min in an electric thermos bottle, washed in distilled water, incubated by 0.3 % H₂O₂ for 30 min and washed in 0.05 M TBS (pH 7.6). The sections were incubated first with anti CD31 antibody (abcam, UK) for 12 hrs at 4°C, and washed in TBS, followed by incubation with secondary anti-rabbit antibody binding HRP (Dako Ltd., Japan) for 2 hrs, and washing in TBS. The final reaction product was developed for 15 min with 3, 3'-diaminobenzidine (DAB) substrate (DAB kit, Dako Ltd., Japan). The sections were washed in TBS and then counterstained with hematoxylin and washed again in tap water. They were dehydrated through a graded series of methanol for 5 minutes in each and mounted in Enteran new (mounting agent) before mounting under a cover slip.

Some paraffin sections were deparaffined and washed in 0.01 M PBS. They were retrieved by the same method above. The sections were incubated first with anti LYVE-1 antibody (RELIAtech GmbH, German) for 4 hrs at 4°C, and washed in TBS, followed by incubation with polyclonal swine anti-rabbit immunoglobulins/AP (Dako Ltd., Japan) for 2 hrs, and washed in TBS. The final reaction product was developed for 1 hr with BCIP/NBT (5-Bromo-4-Chloro-3-Indoxyl Phosphate/Nitro Blue Tetrazolium Chloride) Substrate System (Dako Ltd., Japan). The sections were washed in TBS and then counterstained with kernechtrot or methylgreen and washed again in tap water. They were mounted in glycelgel (Aqueous mounting medium) before mounting under a cover slip. Cryosections were stained in a similar procedure, except for the antigen retrieval. Cryosections for anti CD31 and LYVY-1 antibodies were stained with a similar procedure for paraffin sections, except for the antigen retrieval. Negative controls were done by

omitting primary antibodies.

Paraffin sections and cryosections were subjected to double immunostaining with anti CD 31 and anti LYVE-1 antibodies in this order.

Some cryosections without fixation were used for electron microscopic immunohistochemistry. They were stained with anti LYVE-1 antibody to detect the lymphatics according to the pre-embedding method. Cryosections on glass slides were dried in air, washed with 10% sucrose in PBS, reacted with 0.3% H₂O₂ for 30 mins, and incubated with anti LYVE-1 antibody at 4°C for 12 hrs. They were washed with 10% sucrose in PBS, incubated with HRP conjugated anti-rabbit IgG (H + L chain) (Medical & Biological Laboratories Co., Ltd., Japan) for 4°C for 12 hrs, and washed with 10% sucrose in PBS. They were fixed with 0.5% glutaraldehyde in 0.1M phosphate buffer, pH7.4, for 5 min, and washed with 10% sucrose in PBS. They were reacted with 0.02% DAB in 0.05 M in trisphosphate buffer, pH7.6, for 30 min at room temperature, followed by incubation with 17μL 30% H₂O₂ in 100 mL 0.02% DAB in 0.05 M trisphosphate buffer (pH 7.6) for 5 min and washing in 10% sucrose in PBS. Then they were postfixed with 2% osmium tetroxide for 1 hr. They were dehydrated in grades of alcohol, embedded in Quetol 812 and cut 70 nm in thickness. The sections were observed with no stain with the transmission electron microscope (JEM-1210, JEOL Ltd., Japan).

Specimens fixed with glutaraldehyde and osmic acid were ultrathinly cut and double stained with uranyl acetate and lead citrate according to the conventional method.

Counting vessels in the wounds and intact dermis

Granulation tissue was input into the computer by Adobe Photoshop Element 2.0 and the area was calculated by Scion Image Beta 4.02. The number of new blood and lymphatic vessels in granulation tissue was counted by observation through the light microscope with magnification 400. Five sections per days 5, 7, 9, 11, 13, and 15 after wounding were used, all from the center of the wounds. In the same way, the number of blood and

lymphatic vessels in normal dermis (1 mm in width) of skin harvested at wounding was counted.

Statistical analysis

Data are expressed as mean \pm SD. The number of new blood and lymphatic vessels in wounds and the number of blood and lymphatic vessels in normal dermis were compared by Mann-Whitney test using SPSS for Windows Version 10.1 (SPSS Inc., USA) and differences were considered significant at $p < 0.05$.

Results

Wound area (Fig. 2)

The ratios of wound area on day 0 to day 15 to initial wound area on day 0 were calculated. Wound area increased and peaked on day 1 and was 1.15 ± 0.33 times as large as on day 0. After this, the wound area decreased to 0.9 times on day 4 as compared to day 0. After day 4, the wound area was rapidly reduced to 0.09 ± 0.14 times on day 10 as compared to day 0. The wound scarred by day 15.

Quantity of exudate (Fig.2)

A hydrocolloid dressing covered the wound from day 0 to day 1 for about 24 hours and absorbed 0.22 ± 0.066 g of exudate per wound. The amount of exudate collected during this period was used as the exudate weight for day 0. Ratios of exudate weight on day 0 to day 14 to initial exudate weight on day 0 were calculated. The amount of exudate absorbed in the dressing peaked on day 1 and was 1.00 ± 0.27 times as large as on day 0. After this, the amount of exudate rapidly decreased to 0.25 ± 0.25 times on day 6 as compared to day 0. It became difficult to measure the quantity of exudate absorbed in the dressing after approximately day 11 when the wound surface was completely covered with the new epithelium.

Quantities of new blood and lymphatic vessels in granulation tissue (Fig 3)

Granulation tissue was not formed on day 3 after wounding, but was observed by 5 days after wounding. The number of new blood vessels stained brown with anti CD31 antibody was $146.0 \pm 57.9/\text{mm}^2$ on day 5, $160.3 \pm 100.4/\text{mm}^2$ at the peak on day 7, and decreased gradually to $52.3 \pm 16.5/\text{mm}^2$ in the scar on day 15, which was almost equal to the number on intact dorsal skin of 8-week-old mice, $41.4 \pm 6.8/\text{mm}^2$. New lymphatic vessels were observed in the granulation tissue for the first time on day 7 after wounding. The number of new lymphatic vessels stained purple with anti LYVE-1 antibody was $3.4 \pm 1.9/\text{mm}^2$ on day 7, $51.5 \pm 13.8/\text{mm}^2$ at peak on day 11, and

decreased to $37.1 \pm 11.3/\text{mm}^2$ on day 15, which was almost equal to the amount measured in intact dorsal skin of 8-week-old mice, $21.9 \pm 7.8/\text{mm}^2$.

Histological observations

On days 1 and 3 (Fig. 4) during the inflammation phase, there was a concave space between the surrounding skin and wound floor, which was filled with exudate. No granulation tissue was observed. New epithelium formed along the wound edge. Lymphatic vessels in the surrounding dermis were expanded. This may be due to absorption of exudate leaking from the remaining blood vessels.

On day 5 (Fig. 5) granulation tissue began to form and filled the concave space of the wound. Many new blood vessels stained with anti CD 31 antibody appeared throughout the granulation tissue. Red blood cells, neutrophils and macrophages as inflammatory cells, and fibroblasts were observed in the granulation tissue. No lymphatic vessels were present in the granulation tissue. New epithelium elongated and covered about one fourth of the wound surface.

On day 7 (Fig. 6) after wounding, many new blood vessels were observed throughout the granulation tissue. New lymphatic vessels stained with anti LYVE-1 antibody were only observed near the wound edge in the periphery of the granulation tissue. But no lymphatic vessels were present in the center of the granulation tissue.

On day 9 (Fig. 7) after wounding, lymphatic vessels were observed in the periphery of granulation tissue with the transmission electron microscope. Thin endothelial cytoplasm protruded into the granulation tissue. No lymphatics were observed in the center of the granulation tissue. New epithelium almost completely covered the wound surface.

On day 11 (Fig. 8) lymphatic vessels were present in the center of the granulation tissue and were distributed in the whole granulation tissue. A few new lymphatics expanded into the granulation tissue as they absorbed intercellular fluid. The whole wound surface was completely covered with new epithelium.

On days 13 and 15 (Fig. 9) lymphatics were distributed in the scar. Some of them were connected with the lymphatics present in the dermis or subcutaneous area of the surrounding skin, but we did not observe that the lymphatics in the scar continued to the lymphatics in the fascia under the scar covering the muscle. Moreover, the expanded lymphatics were not observed as on day 11. So the distribution and aspect of the lymphatics in the scar were similar to that in the intact dermis. The distribution and aspect of the blood vessels in the scar were similar to that in the intact dermis.

Indian ink injected into the granulation tissue (Fig. 10)

Since the quantity of the exudate that leaked from the wound surface decreased extremely until 7 days after wounding when the new lymphatics were first observed in the granulation tissue, this suggested that new lymphatics did not take part in the absorption of the exudate, and thus we injected Indian ink into the granulation tissue or the concaved space of the wound (see fig. 4) on days 3, 5, and 7 to determine whether the exudate flows in only one way, from within the granulation tissue or the concaved space to its surface. Interestingly Indian ink injected into the granulation tissue scarcely spread into the dermis, subcutaneous or fascia surrounding the granulation tissue but some leaked from the surface of the granulation tissue, and most of the ink was retained in the granulation tissue. On the other hand, Indian ink injected into the intact skin, dermis or subcutaneous layer diffuses and is absorbed by the lymphatics in the skin.

Discussion

Many new blood vessels appeared in the whole granulation tissue on day 5 after wounding. The number of vessels peaked around $150/\text{mm}^2$ on day 9 and the number of vessels was reduced to $65/\text{mm}^2$ on day 15, which was almost equal to the number of vessels in normal dermis. On the other hand, new lymphatics, about 3 lymphatics/ mm^2 , were detected only at the wound edge on day 7 in the periphery of the granulation tissue near the wound edge. Then, new lymphatic vessels were gradually formed by the extension of the existing lymphatic vessels in the surrounding intact skin into the granulation tissue, as in previous studies.²⁴⁻²⁶ It takes about 5 days for the new lymphatics to reach into the center of the granulation tissue. The 2-day time lag between the appearance of blood and lymphatic vessels in the granulation tissue in the present study is the same as in the research by Paavonen et al.⁴ but is shorter than the studies by Hong et al.⁷ and Bellman and Oden⁶ in which the growth of newly formed lymphatic vessels into the granulation tissue occurred approximately one week later than the sprouting blood vessels. This order, that is when new blood vessels first appear and new lymphatic vessels appear second, is the same as that in the development of blood and lymphatic vessels.^{25, 27} Since the importance of this order is not yet clear, the regenerative relationship between the blood and lymphatic vessels should be determined.

The amount of exudate decreased to one third of the initial amount up to 5 days after wounding when new blood vessels began to form and no new lymphatic vessels were present in the granulation tissue, and to one fifth of the initial amount after 7 days when new lymphatics appeared in the granulation tissue. This decrease of exudate is almost the same as that in our previous study that measured exudate leaking from the wound surface,¹¹ although lymphangiogenesis was not determined in the previous study. Moreover, the peaks in the number of new blood vessels and new lymphatic vessels are on days 7 and 11, when it is difficult to measure the amount of exudate, although the

peaks are slightly different compared to another study⁴. Since the beginning and peak of appearance of new lymphatics occurs after the amount of exudate is very low, the decline of exudate may not be related to the formation of new lymphatics in the granulation tissue. Moreover, the new epithelium almost covers the wound surface in 9 days when the number of new blood vessels almost peaks and a few new lymphatics appear. This suggests that the decline of exudate from the wound surface is due to reducing the acute inflammation by hemostasis and decreasing vascular permeability during the inflammation phase before granulation tissue formation phase, not directly due to forming new lymphatics. However, when the wound surface is completely covered with new epithelium on day 11 after wounding, the number of blood vessels in the granulation tissue decreases rapidly but new lymphatics are rapidly formed and the number of lymphatics peaks, and then gradually decreases to the same number of the lymphatic in the normal skin on day 15 when the wound is full of the scar tissue. Moreover, impaired lymphangiogenesis in cutaneous wound healing due to administration of rapamycin²⁸ or in diabetic mice (*db/db*)²⁹ induces macroscopic lymphedema and delay of wound closure, respectively, although Hong et al. report that no impairment of wound closure rates is observed after treatment with anti-VEGFR-2 blocking antibody DC101, which completely inhibits lymphangiogenesis. This may indicate that the increasing lymphatics during the late phase of granulation tissue formation, when the wound surface is almost covered with new epidermis, play a role in draining interstitial fluid in the granulation tissue or scar produced from the blood vessels and thus promote the late phase of the wound healing process or the formation of fibrous granulation tissue or scar.

According to the results of Indian ink injected into the wound on days 3, 5, and 7 during the inflammatory phase and early granulation tissue formation phase, exudate into the wound from the existing or new blood vessels does not seem to be absorbed by the existing lymphatic in the surrounding skin and a few new lymphatics in the periphery of

the granulation tissue, but leaks from the wound surface. On the other hand, Indian ink injected into the intact skin, dermis or subcutaneous layer diffuses and is absorbed by the lymphatics in the skin. This indicates that some exudate leaked from the existing blood vessels into the surrounding intact skin is absorbed into the existing lymphatics in the intact skin, and exudate that flows into the wound area probably does not flow backward into the intact skin, but rather leaks from the wound surface. There is a strong one-way flow from the surrounding tissue to the wound area in the inflammatory and granulation tissue formation phases and to the wound surface. This phenomenon may be important for wound healing because the important role of inflammatory exudate is to maintain the moisture circumference of the wound by covering the wound surface, to wash out debris including inflammatory cells, and to supply fresh growth factors.^{12, 13, 15-17}

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research from Japan (No. 19592433).

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Figure Legends

Figure 1. Two hydrocolloid dressings (HD) absorbing exudate for 24 hours. Quantity of exudate (g) = weight of HD with absorbed exudate on the following day – weight of HC on day one.

Figure 2. Graph showing the ratios (mean \pm SD) of wound area and quantity of exudate by days after wounding. Note the rapid decrease of the amount of exudate.

Figure 3. Graph showing the mean number and standard deviation of new blood and lymphatic vessels per mm² in the granulation tissue and scar by days after wounding, and the mean number without the standard deviation of blood and lymphatic vessels per mm² in the intact dorsal skin of 8-week-old mice. *P<0.05: Number of new blood vessels in granulation tissue compared with that in intact skin. **P<0.05: Number of new lymphatic vessels in granulation tissue compared with that in intact skin.

Figure 4. Wound perpendicular sections on day 3 after wounding. No granulation tissue is formed in the wound area (WA). Wound edge (WE) is demarcated by the divided end of epithelium (E) and dermis (D). Note the lymphatics in D stained purple by LYVE-1 are strongly dilated (arrows in a and close up picture, b).

Figure 5. Wound horizontal sections on day 5 after wounding. There are many new blood vessels stained brown with CD31 in the granulation tissue (GT) but there are no lymphatic vessels in GT (a and c). There are a few dilated lymphatics stained purple by LYVE-1 around the hair follicle in the intact dermis (b). D: Dermis. Dotted line indicates the wound edge.

Figure 6. Wound perpendicular (a) and horizontal (b) sections on day 7 after wounding. Only a few new lymphatic vessels (purple) have appeared on the peripheral portion of the granulation tissue (GT) (a, b and e), and were slightly expanded (c and e). Note that there are no lymphatics in the center of GT (c), although there are numerous new blood vessels (brown) throughout GT. NT: necrotic tissue.

Figure 7. Photographs of semithin section stained with toluidine blue (a), ultrathin section immunostained with anti-LYVE-1 antibody (b), and ultrathin section double stained with uranyl acetate and lead citrate (c) on day 9 after wounding. These lymphatics (L) are present in the peripheral area of the granulation tissue (GT) and seem to be growing into GT by sprouting of cytoplasmic processes of lymphatic endothelial cells (arrows) .

Figure 8. Wound perpendicular (a) and horizontal (b) sections on day 11 after wounding. Note that new lymphatic vessels (purple) distribute throughout the whole area of granulation tissue (GT), suggesting that the new lymphatics grow from the periphery (see Fig. 7) to the center of GT over about 5 days (c and d). Many blood vessels (brown) remained in GT. Dotted lines indicate the demarcation of wound edge.

Figure 9. Wound perpendicular (a) and horizontal (b) sections on day 13 after wounding. A lot of lymphatics (arrows) run from the subcutaneous (a) and dermis (D) (b and c) around the granulation tissue (GT) to GT. There are very few lymphatics in the inferior part or fascia (*). GT seems to be almost a scar containing a large amount of collagen and a lot of blood and lymphatic vessels. Dotted lines indicate the demarcation of wound edge.

Figure 10. Photographs (a and b) and histological sections (c-f) of wound injected with Indian ink two hours later after ink injection on day 7 after wounding. Macroscopically, Indian ink (arrows) injected into the wound or granulation tissue (GT) remains in the wound and does not spread into the surrounding (a and b). Histologically, although ink almost leaks from the wound or GT, a small amount of ink is present in GT (d), but no ink is observed in peripheral portion of GT (e and f), nor in the lymphatics. D: dermis.

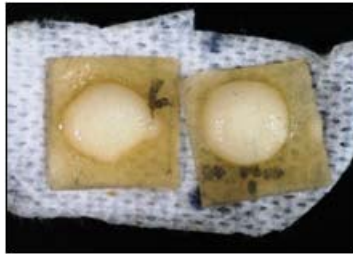


Figure 1

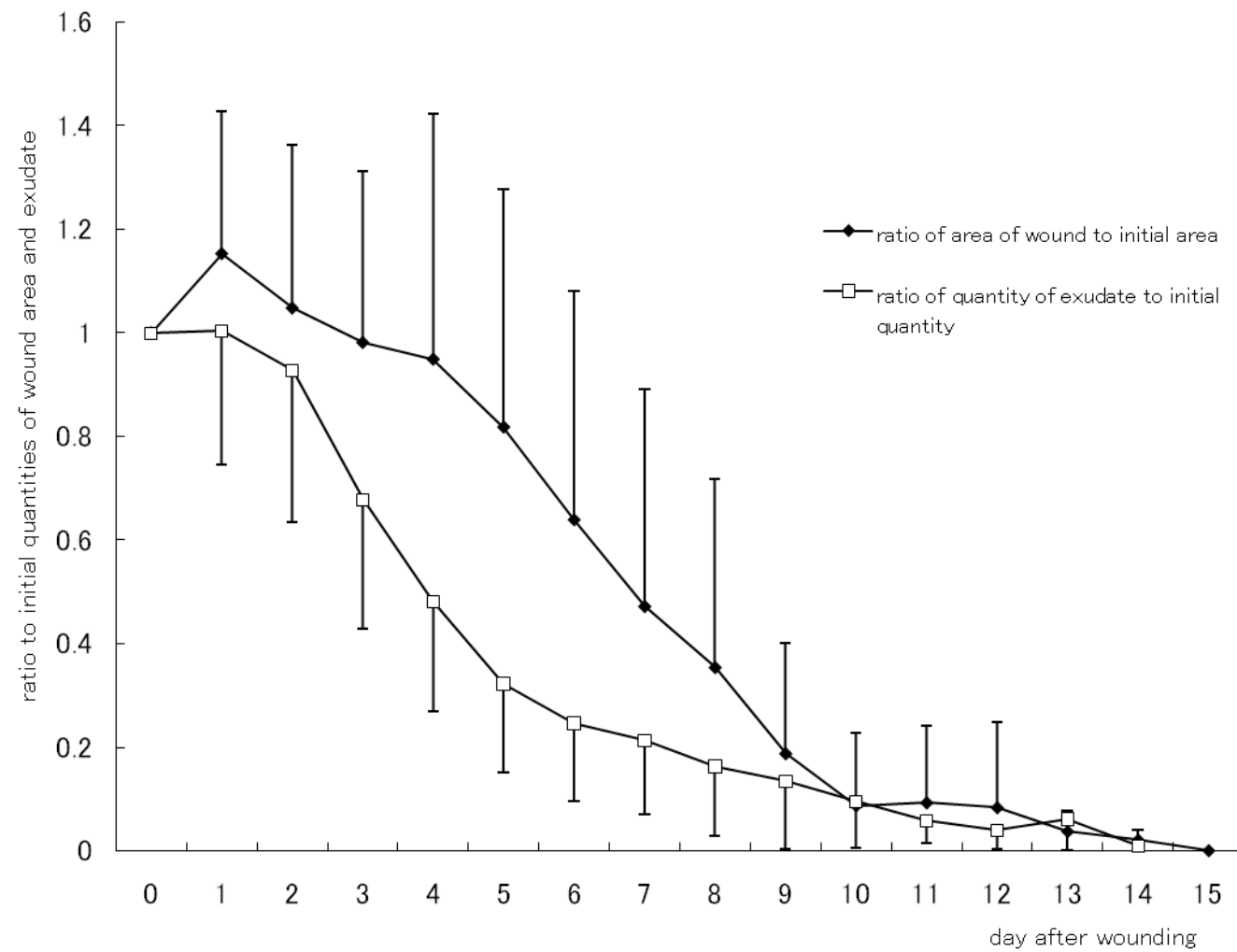


Figure 2

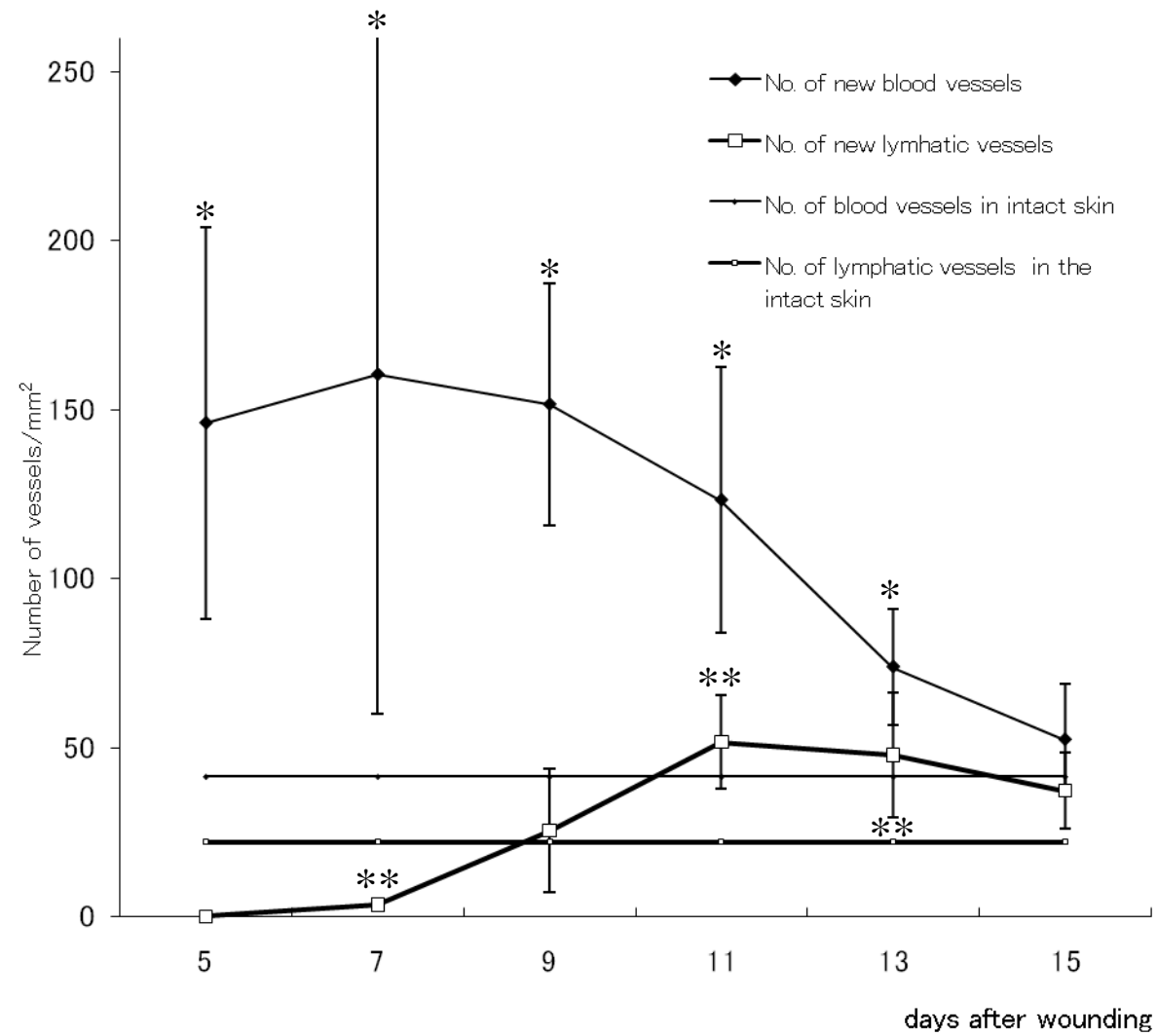


Figure 3

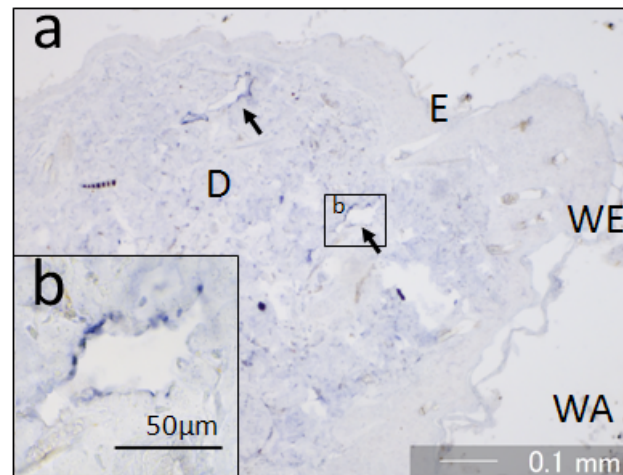


Figure 4

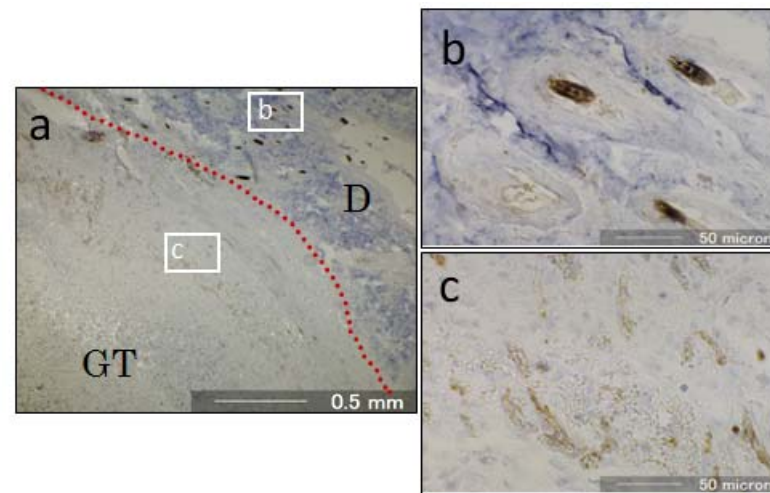


Figure 5

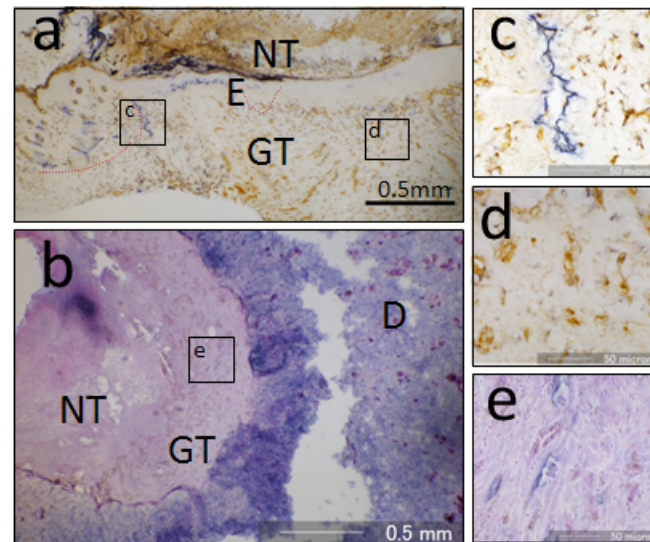


Figure 6

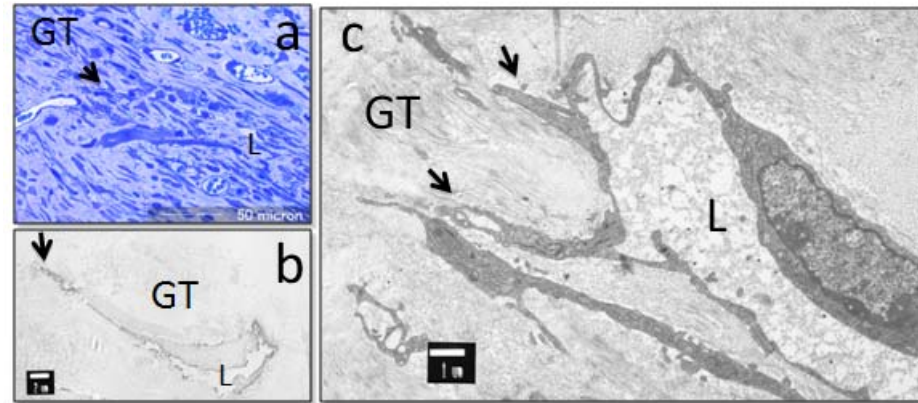


Figure 7

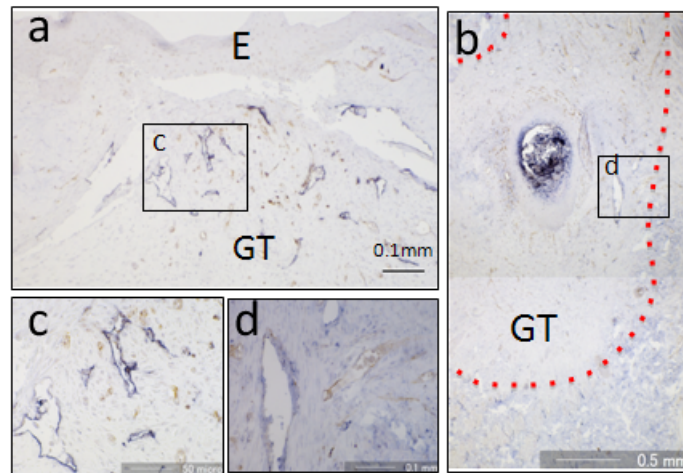


Figure 8

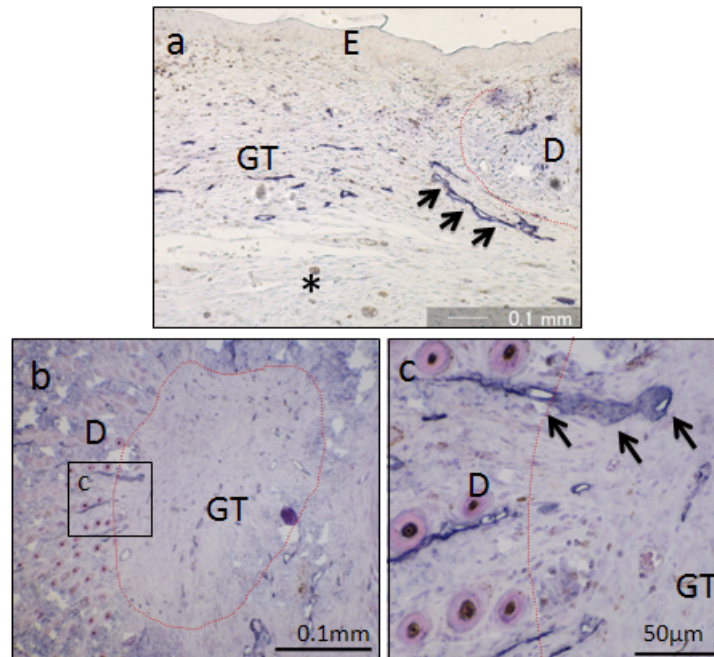


Figure 9

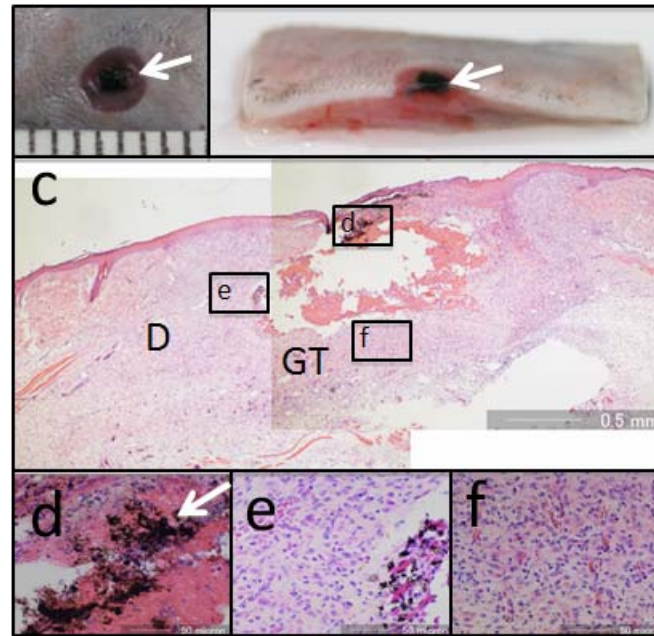


Figure 10