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Changes in the rat subcutaneous connective tissue after saline and histamine injection in relation to fluid storage and excretion*

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Summary. An experimental design was developed for morphometric analysis of the subcutaneous connective tissue after the subcutaneous injection of 0.1 m*l* of saline or a histamine solution (0.01, 0.1 or 1% histamine dihydrochloride in saline). The subcutaneous connective tissue of 4-week-old rats, originally 170.0 ± 13.6 μm in thickness, swelled 5.2-fold at 15 min, 3.0-fold at 2 h, and 1.2-fold at 6 h after the injection of saline. The total cross sectional area of both blood and lymphatic vessels increased when compared to that at pre-injection (0.0186 ± 0.0030 mm^2 in 6-mm-long specimen), 1.4-fold at 15 min, 2.2-fold at 2 h, and 1.1-fold at 6 h post-injection, while the total number of these vessels increased 1.1-fold at 2 h. The total cross sectional area of lymphatic vessels (0.0006 ± 0.0002 mm^2 in 6-mm-long specimen) alone surged 7.7-fold at 15 min, 4.8-fold at 2 h, and 7.3-fold at 6 h. Collagen fibers were respectively highly, moderately, and mildly disorganized in arrangement at 15 min, 2 h, and 6 h after the saline injection. Histamine elicited an earlier, longer, and more pronounced vasodilatation, particularly at high concentrations. The transvascular permeability of Evans blue increased depending on the concentration of histamine. These findings indicate that the subcutaneous connective tissue has the ability to expand and store a considerable amount of fluid and reversibly returns to normal steady-state conditions by increasing fluid excretion into the blood and lymphatic vessels. It was also strongly suggested that the blood vessels are deeply involved in the excretion and volume regulation of the tissue fluid.

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Introduction

The subcutaneous connective tissue of mammals is composed of multiple layers of thin collagen sheets containing some elastic fibers. These thin layers of connective tissue play an essential role in decreasing the friction between the skin and the underlying tissues such as muscles, bones, and tendons (Kawamata *et al.*, 2003). The subcutaneous connective tissue also functions as a reservoir and pathway for tissue fluids (Reed *et al.*, 1985), and an accumulation of excessive fluid may result in edemas, e.g., histamine release by allergic reactions. The fluid in the subcutaneous connective tissue is maintained at a constant volume and component composition (Reed *et al.*, 1989) by a continuous exchange of fluid and materials between the tissue and the blood and lymphatic vessels. In addition, the subcutaneous connective tissue and its veins are often used for subcutaneous injections and intraveous infusions, respectively, in medical practice. Injected or leaked drug solutions may cause tissue damage due to such factors as high osmolarity, nonphysiological pH, or the drug's own toxicity.

However, the mechanism regulating the volume of tissue fluid and the fate of injected or leaked fluids

remain to be understood fully, and quantitative studies are few. The present study therefore examined changes in the subcutaneous connective tissue after an injection of saline (0.9% NaCl) or a histamine solution for simulating allergic reactions and quantitatively investigated the thickness of the subcutaneous connective tissue and the cross sectional area of blood and lymphatic vessels by morphometric analysis.

Materials and Methods

Sixty-five 4-week-old Wistar male rats (93.8 ± 15.7 g) were used in this study. Young animals were chosen because they have little adipose tissue in the subcutaneous connective tissue. Adipose tissue may enhance errors due to individual differences and a rich capillarity. This study was approved by the Committee of Research Facilities for Laboratory Animal Sciences, Hiroshima University.

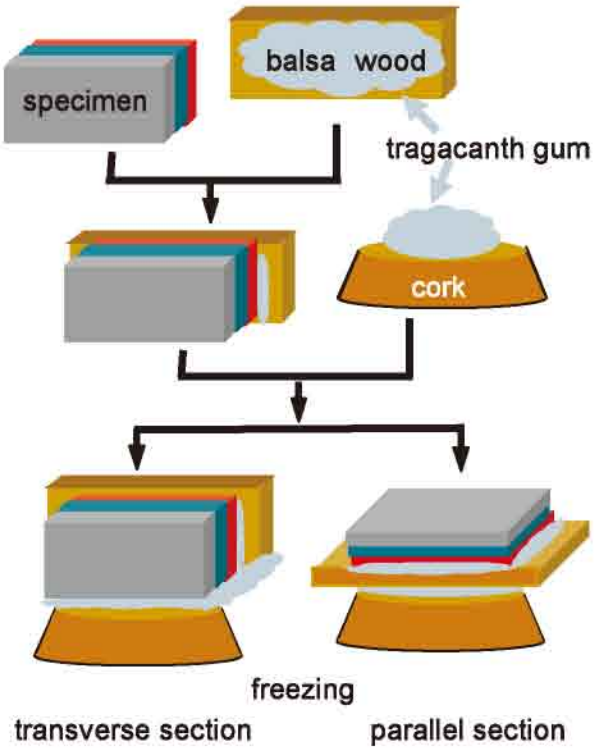


Fig. 1. A schematic drawing of the process for the preparation of specimens. A specimen was adhered to balsa wood (1 mm thick) coated with 6% tragacanth gum. The specimen and balsa wood were placed on a cork disk and supported by tragacanth gum. They were frozen together in isopentane cooled in liquid nitrogen.

Subcutaneous injection

The rats were anesthetized with diethyl ether and the abdominal area shaved with electric clippers. Four sites (upper right and left, lower right and left) on the abdomen of each animal were used injections. The abdominal skin was lifted up with a pair of forceps and a 26 gauge injection needle was inserted into the subcutaneous connective tissue. After confirming that the tip of the needle was in the connective tissue — the tip remained movable after releasing the lifted skin — 0.1 m*l* of either saline or a histamine solution (0.01, 0.1 or 1% histamine dihydrochloride in saline) was injected into the subcutaneous connective tissue, and the injected sites were marked with a felt pen for the convenience of sampling. Uninjected (normal) sites on the abdomen were used as the "pre-injection" sites.

Preparation of histological specimens

The abdominal wall from the epidermis to parietal peritoneum (referred to as the skin-muscle tissue) was removed and fixed to balsa wood (1 mm thick) coated with 6% tragacanth gum to avoid tissue deformity. The specimen and balsa wood were placed on a cork disk (18–22 mm in diameter and several millimeters in thickness) in such a position that the specimen could be cut in a plane either transverse (perpendicular) or parallel (horizontal) to the skin surface. The specimen and balsa wood were frozen together in isopentane cooled in liquid nitrogen (Fig. 1). Three semi-serial transverse sections (10 μm in thickness with 0.5 mm between adjacent sections) from each site and parallel sections (20 μm) were cut, stained with, either hematoxylin-eosin or with aldehyde fuchsin-Masson Goldner, or immunohistochemically, and observed with an Olympus B51 light microscope (Olympus, Tokyo).

Immunostaining

Hematoxylin-eosin staining is known to be inappropriate for detecting small blood and lymphatic vessels. Accordingly, sections were stained using an antibody against PECAM-1 (platelet endothelial cell adhesion molecule; identical to CD31). This antibody (550300, Becton Dickinson Biosciences, San Jose, CA, USA) clearly stains both blood (Scholz and Schaper, 1997)

and lymphatic endothelia, though lymphatic vessels are stained less intensely than blood vessels (Sauter *et al.*, 1998; Ebata *et al.*, 2001; Schacht *et al.*, 2004). To distinguish blood and lymphatic vessels, we stained only lymphatic vessels with an antibody against podoplanin (P-1995, Sigma, St. Louis, MO, USA), a membranous protein found in podocytes in renal glomeruli (Breiteneder-Geleff *et al.*, 1997). It is also expressed in the lymphatic endothelium and is used as a marker for the identification of lymphatic vessels (Breiteneder-Geleff *et al.*, 1999; Matsui *et al.*, 2003; Kato *et al.*, 2006).

Frozen sections were air dried, fixed in cold acetone for 10 min, and rinsed in 0.01 M phosphate-buffered saline (pH 7.4; PBS) once for 5 min. Sections were treated with 0.3% hydrogen peroxide in methanol for 20 min for the inactivation of endogenous peroxidase. After two rinses with PBS for 5 min each, nonspecific binding sites were blocked for 30 min with a blocking solution containing 1% normal horse serum or 1% normal goat serum in PBS, depending on the secondary antibodies to be used. After blotting the blocking solution, the sections were incubated with either an anti-PECAM-1 monoclonal antibody (1: 250) or anti-podoplanin polyclonal antibody (1: 3000) for 2 h at room temperature. Sections were rinsed with PBS 3 times for 5 min each and incubated for 1 h with secondary antibodies, i.e., horse biotinylated anti-mouse IgG (1: 250; BA-2001, Vector, Burlingame, CA, USA) or goat biotinylated anti-rabbit IgG (1: 100; BA-1000, Vector). Then the sections were rinsed with PBS twice for 5 min each and incubated with the streptavidin-biotin complex (1: 50; Elite ABC, Vector) for 30 min. Immunoreactivity was detected with 0.01% hydrogen peroxide and 0.05% 3, 3'-diaminobenzidine tetrahydrochloride in a 0.05 M Tris-HCl buffer (pH 7.2), followed by staining with eosin, after which the sections were dehydrated, cleared with xylene, and mounted. Negative controls incubated with PBS instead of the primary antibodies were all negative.

To observe the blood and lymphatic vessels separately in the same sections, double immunostaining was performed using anti-PECAM-1 and anti-podoplanin antibodies. First, lymphatic vessels were stained with the anti-podoplanin antibody as mentioned above for 2 h, then rinsed and incubated with a biotinylated secondary antibody for 1 h, rinsed, incubated with alkaline phosphatase-labeled avidin-biotin complex (1:100; AK-5001, Vector) for 30 min, and rinsed again, and immunoreactivity was detected with a solution containing 0.1% Naphthol AS-MX phosphate disodium, 0.1% Fast blue RR, and 5% N, N-dimethylformamide in a 0.1 M Tris-HCl buffer (pH 9.2) or alkaline phosphatase substrate kit (SK-5400, Vector). Second, blood vessels were immunostained with the anti-PECAM-1 antibody as above and visualized with a solution containing 0.02% 3-amino-9-ethylcarbazole, 0.006% hydrogen peroxide, and 5% dimethyl sulfoxide in a 0.05 M acetate buffer (pH 5.0).

Preparation of specimens for electron microscopy

Electron microscopy was employed mainly to clarify whether or not vascular walls have smooth muscles. Skin-muscle tissues from 5 rats were dissected in 3% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) and immersion fixed in the same fixative overnight at 4℃. Then specimens were postfixed in 1% OsO_4 in a 0.1 M phosphate buffer (pH 7.4) for 4 h at 4℃, rinsed with 10% saccharose 3 times for 10 min each, and stained *en bloc* with 3% uranyl acetate for 2 h at room temperature. Specimens were dehydrated with an ascending series of ethanol and embedded in epoxy resin. Ultrathin sections (0.1 μm) were cut, stained with uranyl acetate and lead citrate, and observed with a JEM-1200EX electron microscope (JEOL, Tokyo).

Morphometric analysis

Transverse sections were photographed with a DP 70 digital camera (Olympus, Tokyo), and a montage image of each specimen was produced. In the present study, the layer of the subcutaneous connective tissue between the skin muscle and the abdominal muscles was used for measurement because, the superficial boundary of the subcutaneous connective tissue without the skin muscle, was sometimes vague. The boundary of the subcutaneous connective tissue of each sample was traced by the same operator on the montage image. The subcutaneous connective tissue was trimmed using Photoshop version 6.0 (Adobe, San Jose, CA, USA) and then cut at both ends to make it a constant length (6 mm in tissue length) using the computer software Image J version 1.33u (National Institutes of Health, Bethesda, MD, USA). For specimens before injection, the central 6-mm-long portion of each image was used. For samples from sites injected with saline or a histamine solution, the thickest point of the subcutaneous connective tissue was determined, and 3-mm-long portions at both sides (total 6 mm) were selected for measurement.

Thickness of the subcutaneous connective tissue

For measurements of thickness, the area of the trimmed subcutaneous connective tissue was measured and divided by its width (6 mm).

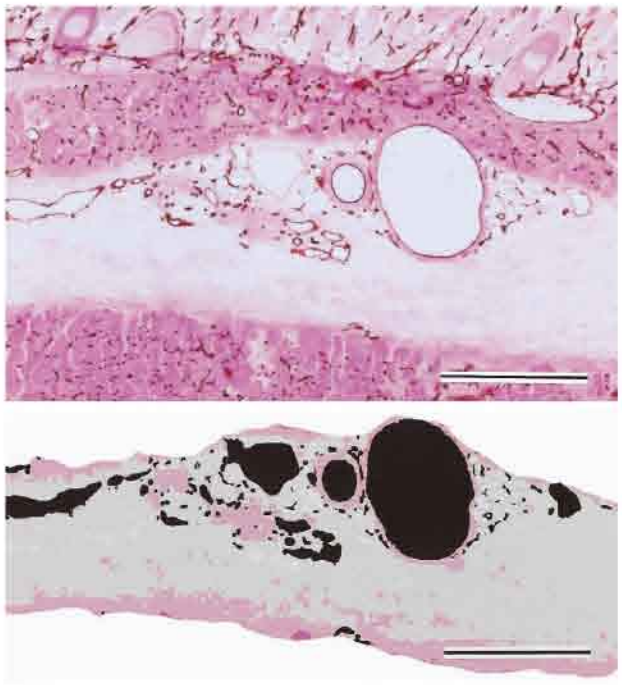


Fig. 2. A transverse section of skin-muscle tissue 2 h after an injection of saline. The subcutaneous connective tissue is sandwiched between layers of the skin muscle (upper) and abdominal muscles (lower). Bars = 500 μm. **a:** A section stained with the anti-PECAM-1 antibody and eosin. The blood and lymphatic vessels including small capillaries are stained. **b:** An image of the subcutaneous connective tissue in panel **a**. The blood and lymphatic vessels were painted black to measure the total cross sectional area. Both blood and lymphatic vessels were detected and converted to a black color with excellent fidelity.

Total cross sectional area of the blood and lymphatic vessels

Using montage images, a given vessel which was typically stained positively for PECAM-1 was chosen in each subcutaneous connective tissue. Immuno-positive regions stained the same color were automatically selected and had their color altered to black by the Photoshop software. The lumini of stained blood and lymphatic vessels — except those not automatically changed to black — were hand painted with black on a computer. The total black area in 6-mm-long sections of subcutaneous connective tissue was calculated (Fig. 2). Thirty-six sections per group (3 animals, 4 sites in each rat, 3 sections per site) were used for measurements. Data of 4 sites were averaged in each animal and further analyzed statistically. In addition, the number of blood and lymphatic vessels was manually counted and compared before and at 2 h after the injection using twelve sections, i.e., one middle section per site.

Total cross sectional area of the lymphatic vessels

Lymphatic vessels alone were stained with the anti-podoplanin antibody, and total cross sectional area was measured in the same manner as above.

Evaluation of transvascular Evans blue leakage

Evans blue is known to bind with albumin in blood plasma. This dye has been used to measure circulating blood volume and to evaluate vascular permeability (Taherzadeh *et al.*, 1998). We employed it to assess the transvascular leakage. Eleven rats were intravenously injected with 1 m*l* of 1% Evans blue in saline via the tail. Fifteen minutes later, either saline or a 0.01, 0.1, or 1% histamine dihydrochloride solution was injected into the subcutaneous connective tissue. After a further 2 h, the animals were sacrificed by the overinhalation of diethyl ether. To minimize the effect of intravascular Evans blue, attention was paid to any decrease in intravascular blood from bleeding while the heart was beating.

Then a skin-muscle tissue specimen 2.0 × 1.0 cm in size was excised. Blue-colored areas were sufficiently large to obtain these specimens in histamine-injected rats. Skin-muscle samples were weighed, dissected, and immersed in 5 m*l* of formamide in air-tight bottles for 24 h at 55℃ for the extraction of Evans blue. Formamide containing the extracted Evans blue was centrifuged for 15 min at 3000 rpm to remove tissue fragments. Light absorbance of supernatants was measured with a UVmini-1240 spectrophotometer (Shimadzu, Kyoto) at 620 nm because this dye mostly absorbs the light of this wavelength. The amount of Evans blue in each sample fluid was calculated using a calibration curve we made and corrected by the tissue weight and dilution of the fluid.

To make the calibration curve, various Evans blue solutions of known concentrations were prepared, and their absorbance of 620-nm light was measured. The absorbance and concentration of Evans blue showed an excellent linear regression from 0 to 6.25 μg/m*l* and the following calibration curve was obtained: $y = 0.084494x + 0.019041$, where y is the concentration of Evans blue (μg/m*l*) and x is the absorption of 620-nm light by a solution.

Some skin-muscle tissue specimens were frozen as mentioned above, cryosectioned, freeze-dried in a refrigerator, and photographed under a dissecting microscope SZX10 (Olympus, Tokyo). During freeze-drying, the muscle layer became whitish and Evans blue was more easily observable.

Statistical analysis

Thirty-six sections in each group (3 animals, 4 sites in each rat, 3 sections per site) were used for measurements of the thickness of the subcutaneous connective tissue and the total cross sectional area of the blood and lymphatic vessels. Twelve sections (one middle section per site mentioned above) were used for counting the number of blood and lymphatic vessels.

Data of 4 sites were averaged for each animal and further analyzed statistically. Eight animals per group were used for measurements of the transvascular Evans blue leakage. Data are describrd as the mean ± SD. Data pre- and post-injections were alalyzed with the F test and Student's unpaired t test. Statistical significance was set at $P<0.05$.

Results

Structure of the normal subcutaneous connective tissue

The rat subcutaneous connective tissue appeared as a thin layer sandwiched between the skin muscle and the abdominal muscles and was composed of loosely arranged thin collagen sheets containing some elastic fibers. Collagen fibers ran parallel to the skin with undulations. Scattered among the collagen sheets were the blood and lymphatic vessels, nerves, and sometimes adipose tissues (Fig. 3). By hematoxylin-eosin staining, large blood vessels were easily observable, whereas lymphatic and small blood vessels were difficult to detect with certainty.

In transverse sections stained with the anti-PECAM-1 antibody, blood vessels ran parallel to the skin, and no blood vessels extended in a straight line away from the abdominal muscles to the dermis. Blood vessels were frequently observed around any nerves and adipose tissue present, and ran at different depths in the subcutaneous connective tissue. Blood and lymphatic vessels varied greatly in size from small to as large as 100 μm in diameter. Blood vessels were usually round in shape and numerous, whereas lymphatic vessels were often irregular in shape and small in number. In sections parallel to the skin, blood vessels extended to form a meshwork

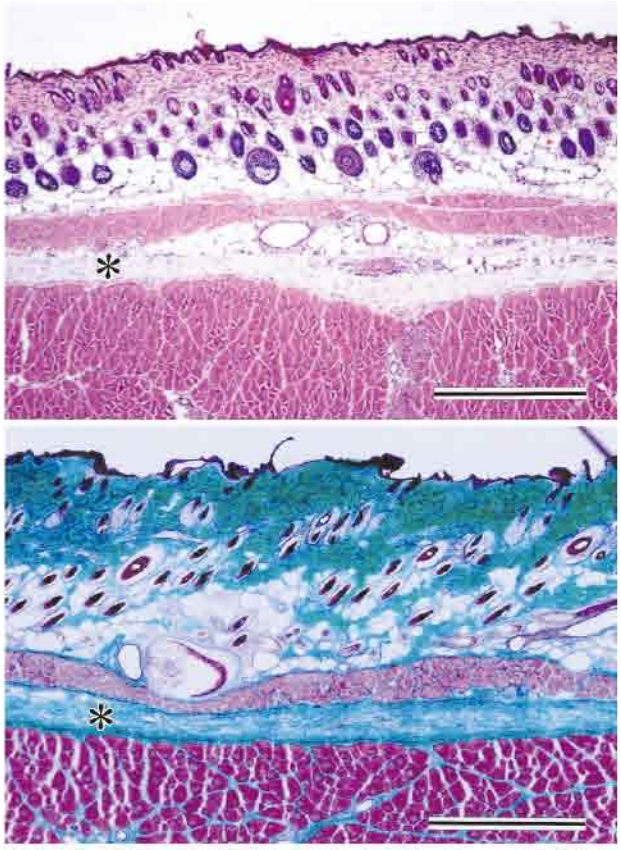


Fig. 3. Sections of normal (pre-injected) skin-muscle tissue showing the epidermis, dermis, skin muscle, subcutaneous connective tissue (asterisks), and abdominal muscles. Bars = 500 μm. **a:** Hematoxylin-eosin staining. **b:** Aldehyde fuchsin-Masson Goldner staining.

arrangement, and the lymphatic vessels tended to run along the blood vessels (Fig. 4).

Changes after saline was injected

The thickness of the subcutaneous connective tissue was significantly increased after the injection but decreased with time (Fig. 5). At 15 min, the arrangement of collagen fibers was highly disorganized and sparse with wide interfiber spaces except for areas in the vicinity of blood vessels, nerves, and epimysium. At 2 h after the injection, deranged collagen fibers were observed. At 6 h after the injection, the arrangement of collagen fibers was mildly disoriented and resembled that before the injection (Fig. 6). The thickness of the subcutaneous connective tissue

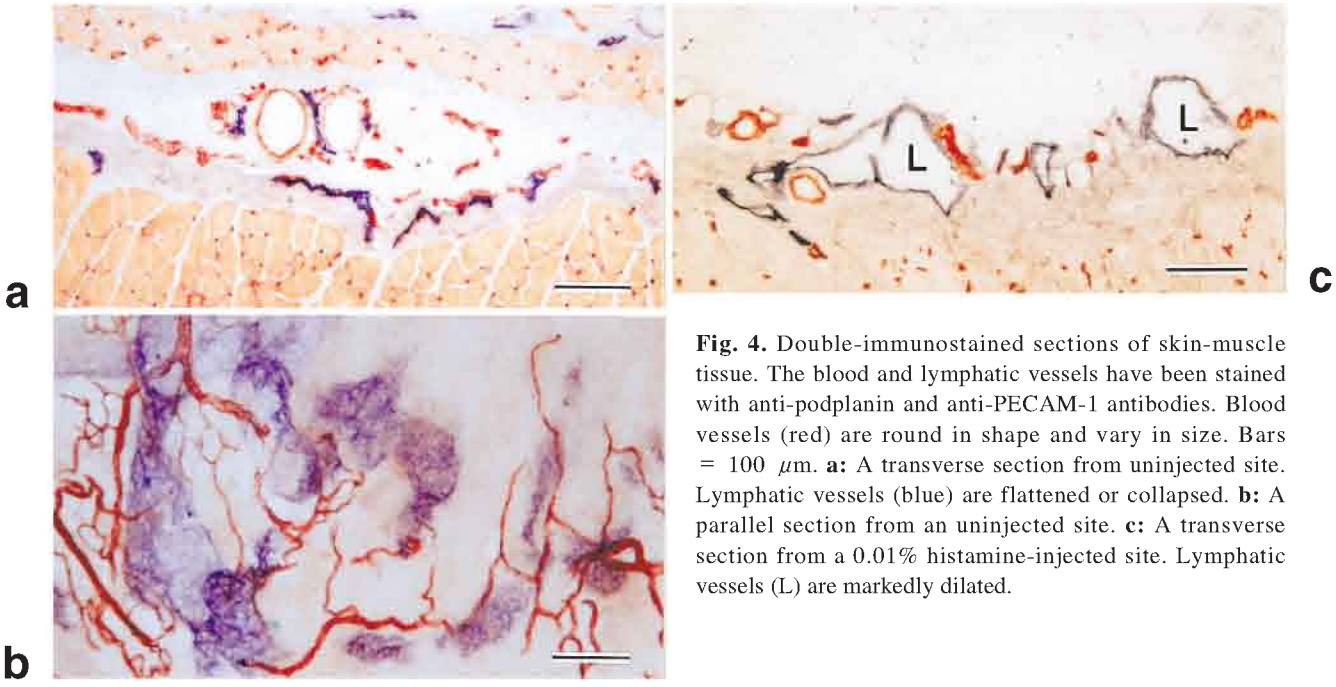


Fig. 4. Double-immunostained sections of skin-muscle tissue. The blood and lymphatic vessels have been stained with anti-podplanin and anti-PECAM-1 antibodies. Blood vessels (red) are round in shape and vary in size. Bars = 100 μm. **a:** A transverse section from uninjected site. Lymphatic vessels (blue) are flattened or collapsed. **b:** A parallel section from an uninjected site. **c:** A transverse section from a 0.01% histamine-injected site. Lymphatic vessels (L) are markedly dilated.

was 170.0 ± 13.6 μm before the injection, and 884.8 ± 70.3 μm (5.2-fold increase) at 15 min, 510.3 ± 163.9 μm (3.0-fold) at 2 h, and 205.6 ± 42.9 μm (1.2-fold) at 6 h after the saline was injected (Fig. 7).

The total cross sectional area of blood and lymphatic vessels in sections of subcutaneous connective tissue 6-mm in length was 0.0186 ± 0.0030 mm^2 before the injection, and 0.0261 ± 0.0059 mm^2 (1.4-fold increase) at 15 min, 0.0413 ± 0.0049 mm^2 (2.2-fold) at 2 h, and 0.0206 ± 0.0009 mm^2 (1.1-fold) at 6 h after the injection (Figs. 8, 9). The numbers of blood and lymphatic vessels were 243.3 ± 43.8 at uninjected sites and 263.3 ± 47.3 (1.1-fold increase) at 2 h after the saline was injected.

The total cross sectional area of the lymphatic vessels was 0.0006 ± 0.0002 mm^2 before the injection, and 0.0046 ± 0.0009 mm^2 (7.7-fold increase) at 15 min, 0.0029 ± 0.0008 mm^2 (4.8-fold) at 2 h, and 0.0044 ± 0.0005 mm^2 (7.3-fold) at 6 h after the injection of saline (Fig. 8).

Changes after histamine was injected

At 15 min after the injection, the subcutaneous connective tissue was much thicker and the arrangement of fibers highly disorganized compared with conditions before the injection of histamine.

0.01% histamine

The thickness of the subcutaneous tissue was 170.0 ± 13.6 μm before the injection, and 1049.3 ± 113.5 μm (6.2-fold increase) at 15 min, 539.3 ± 28.4 μm (3.2-fold) at 2 h, and 260.2 ± 49.4 μm (1.5-fold) at 6 h after the injection (Fig. 7). The total cross sectional area of blood and lymphatic vessels was 0.0186 ± 0.0030 mm^2 before the injection, and 0.0275 ± 0.0001 mm^2 (1.5-fold increase) at 15 min, 0.0371 ± 0.0090 mm^2 (2.0-fold) at 2 h, and 0.0258 ± 0.0044 mm^2 (1.4-fold) at 6 h after the injection. The total cross sectional area of lymphatic vessels was 0.0006 ± 0.0002 mm^2 before the injection, and 0.0051 ± 0.0007 mm^2 (8.5-fold increase) at 15 min, 0.0032 ± 0.0002 mm^2 (5.3-fold) at 2 h, and 0.0043 ± 0.0008 mm^2 (7.2-fold) at 6 h after the injection (Fig. 8).

0.1% histamine

The thickness of the subcutaneous tissue was 170.0 ± 13.6 μm before the injection, and 961.2 ± 210.6 μm (5.7-fold increase) at 15 min, 520.5 ± 60.4 μm (3.1-fold) at 2 h, and 323.9 ± 26.5 μm (1.9-fold) at 6 h after the injection (Fig. 7). The total cross sectional area of blood and lymphatic vessels was 0.0186 ± 0.0030 mm^2 before the injection, and 0.0277 ± 0.0034 mm^2 (1.5-

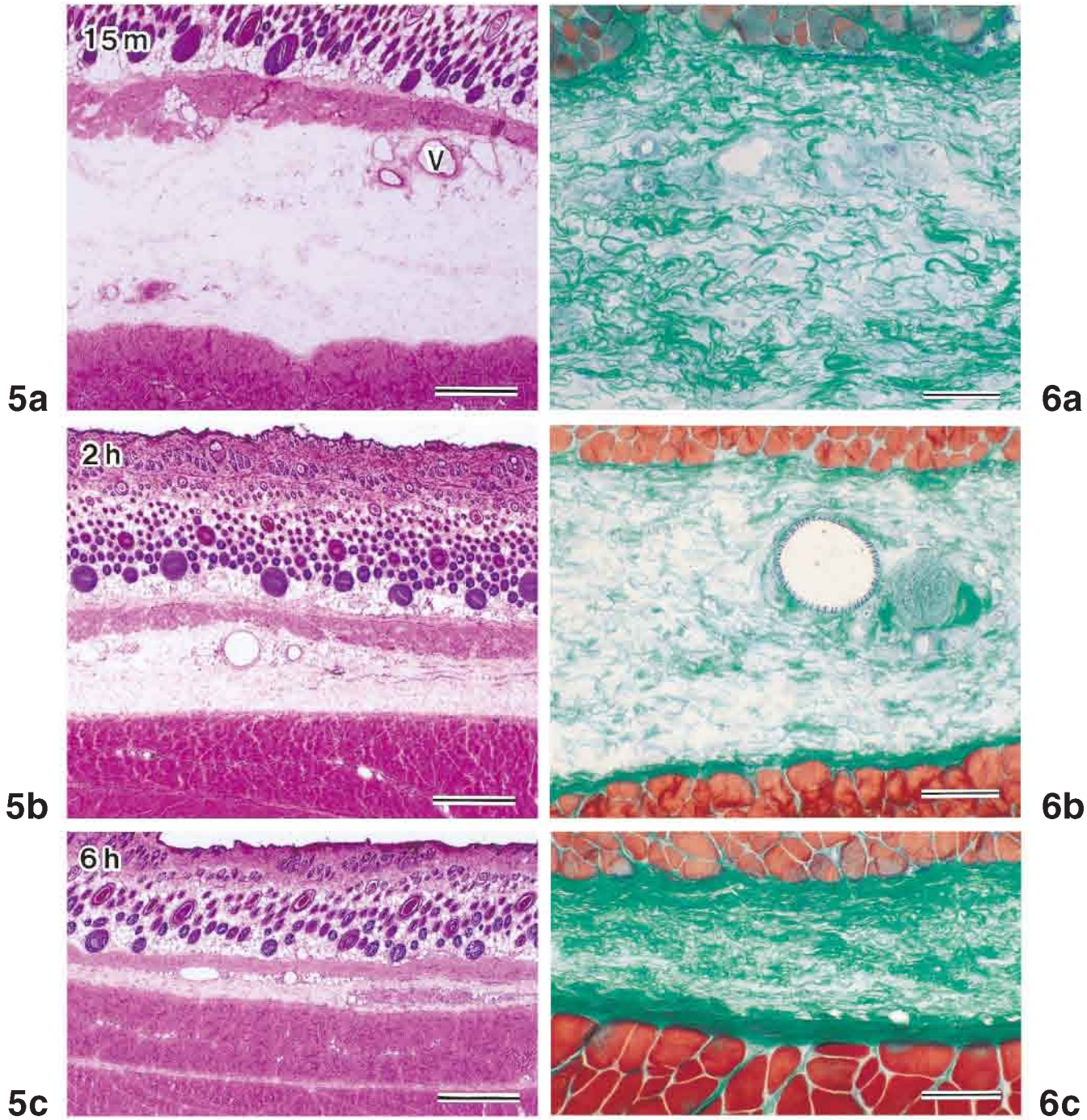


Fig. 5. Sections of skin-muscle tissue after an injection of saline. Hematoxylin-eosin staining. Bars = 500 μm. **a:** At 15 min post-injection. The subcutaneous connective tissue is markedly increased in thickness. Large vessels (V) with a wide lumen are seen. **b:** At 2 h post-injection. **c:** At 6 h post-injection. The subcutaneous connective tissue has nearly returned to a normal condition.

Fig. 6. Sections of skin-muscle tissue after an injection of saline. Aldehyde fuchsin-Masson Goldner staining. Bars = 100 μm. **a:** At 15 min post-injection. Collagen fibers (green) are highly disorganized and interspaces among fibers are wide. **b:** At 2 h post-injection. Deranged collagen fibers are observable. **c:** At 6 h after post-injection. Collagen fibers are mildly disorganized similar to normal conditions.

fold increase) at 15 min, 0.0392 ± 0.0077 mm^2 (2.1-fold) at 2 h, and 0.0369 ± 0.0077 mm^2 (2.0-fold) at 6 h after the injection. The total cross sectional area of lymphatic vessels was 0.0006 ± 0.0002 mm^2 before the injection, and 0.0050 ± 0.0006 mm^2 (8.3-fold increase) at 15 min, 0.0047 ± 0.0005 mm^2 (7.8-fold) at 2 h, and 0.0040 ± 0.0009 mm^2 (6.7-fold) at 6 h after the injection (Fig. 8).

1% histamine

The thickness of the subcutaneous tissue was 170.0 ± 13.6 μm before the injection, and 1106.5 ± 266.4 μm (6.5-fold increase) at 15 min, 1046.5 ± 105.1 μm (6.2-fold) at 2 h, and 546.3 ± 135.2 μm (3.2-fold) at 6 h after the injection (Figs. 7, 10). The total cross sectional area of blood and lymphatic vessels was 0.0186 ± 0.0030 mm^2 before the injection, and 0.0401 ± 0.0082 mm^2 (2.2-fold increase) at 15 min, 0.0394 ± 0.0038 mm^2 (2.1-fold) at 2 h, and 0.0358 ± 0.0053 mm^2 (1.9-fold) at 6 h after the injection. The total cross sectional area of lymphatic vessels was 0.0006 ± 0.0002 mm^2 before the injection, and 0.0058 ± 0.0004 mm^2 (9.7-fold increase) at 15 min, 0.0110 ± 0.0005 mm^2 (18.3-fold) at 2 h, and 0.0071 ± 0.0004 mm^2 (11.8-fold) at 6 h after the injection (Fig. 8).

Extravascular concentration of Evans blue dye in the tissue

At and around the site where histamine was injected, the skin turned blue due to the transvascular leakage of Evans blue. The subcutaneous connective tissue was stained blue, whereas the muscle layers were almost entirely unstained (Fig. 11). Saline alone caused no significant increase in transvascular leakage, whereas histamine caused a significant increase in the extravasation of Evans blue. The concentration of Evans blue before and 2 h after the injection was 8.9 ± 2.9 μg/g tissue at uninjected sites, and 12.4 ± 3.2 μg/g tissue (1.4-fold increase) at saline-injected sites, 25.5 ± 8.5 μg/g tissue (2.9-fold) at 0.01% histamine-injected sites, 30.4 ± 4.6 μg/g tissue (3.4-fold) at 0.1% histamine-injected sites, and 41.8 ± 5.4 μg/g tissue (4.7-fold) at 1% histamine-injected sites (Fig. 12).

Electron microscopy

Blood and lymphatic vessels of varying sizes were clearly observed by electron microscopy. Blood vessels were often found to have smooth muscles, accounting for their vasodilator responses to histamine. Lymphatic vessels were composed of a very thin endothelium without smooth muscles.

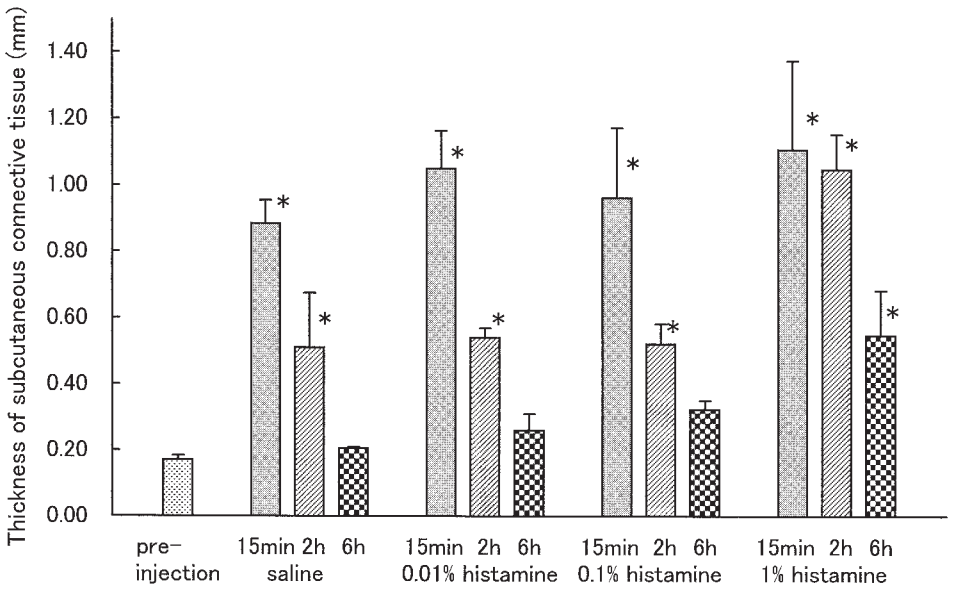


Fig. 7. Thickness of the subcutaneous connective tissue pre- and post-injection (n = 3). *: P<0.05 vs pre-injection

Discussion

In the present study, the time course of the changes in the subcutaneous connective tissue after a subcutaneous injection was quantitatively evaluated by morphometric analysis. It was clearly demonstrated that: 1) the thickness of the subcutaneous connective tissue was significantly increased after the injection but decreased with time, and 2) that the cross sectional areas of both blood and lymphatic vessels were significantly increased after the fluid injection. Although the relationship between cross sectional area and blood flow is not necessarily linear, the significant vasodilator responses of blood vessels are highly likely to reflect an increased excretion of tissue fluid via blood vessels. In fact, Higuchi *et al.* (1999) reported that colloidal particles injected into the subcutaneous connective tissue of the canine hind leg to be more removed by veins (~3% in 5 min) than lymphatic vessels (~0.1% in 45 min).

Changes in the subcutaneous connective tissue after saline injection

After 0.1 m*l* of fluid was injected, the subcutaneous connective tissue markedly increased in thickness and collagen fibers were widely separated. These findings indicate that the subcutaneous connective tissue can expand and store fluid among collagen fibers, perhaps in the way that cotton sheets absorb fluid. The subsequent decrease in the thickness of subcutaneous connective tissue is attributed mainly to the increased drainage of fluid into the blood and lymphatic vessels and partly to the peripheral diffusion of fluid within this connective tissue layer. Although the infusion of fluid into the whole body induces only a slight elevation in interstitial pressure (Wiig and Reed, 1981), the local injection of saline probably results in an elevation of interstitial pressure and a decrease in colloid osmotic pressure because saline contains no proteins. These changes are considered to promote reabsorption from both blood and lymphatic

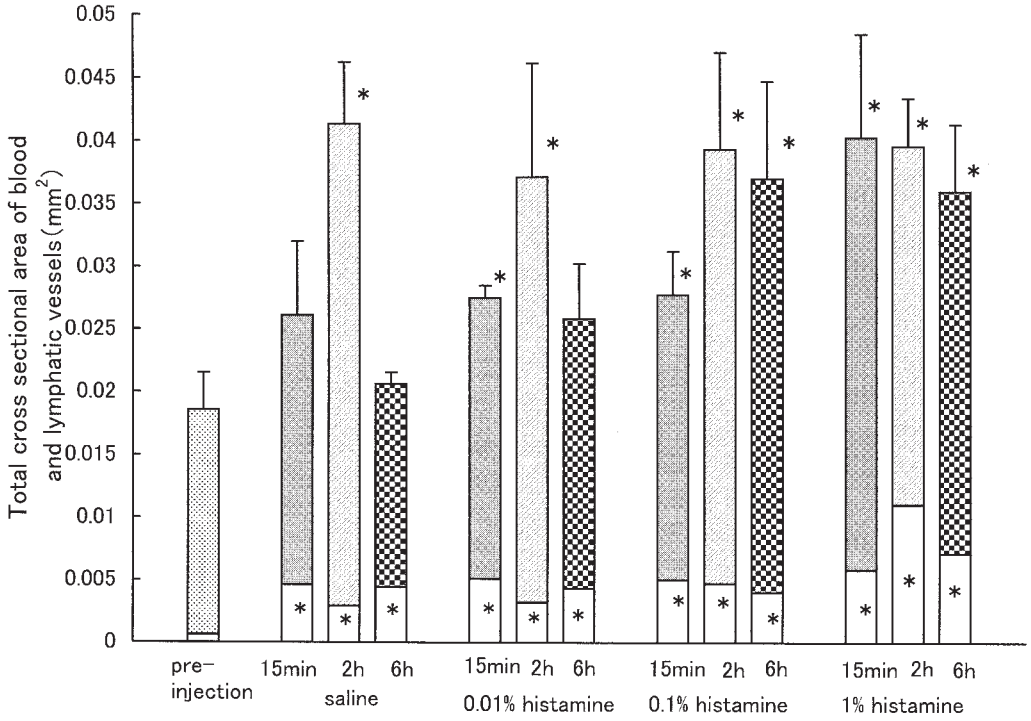
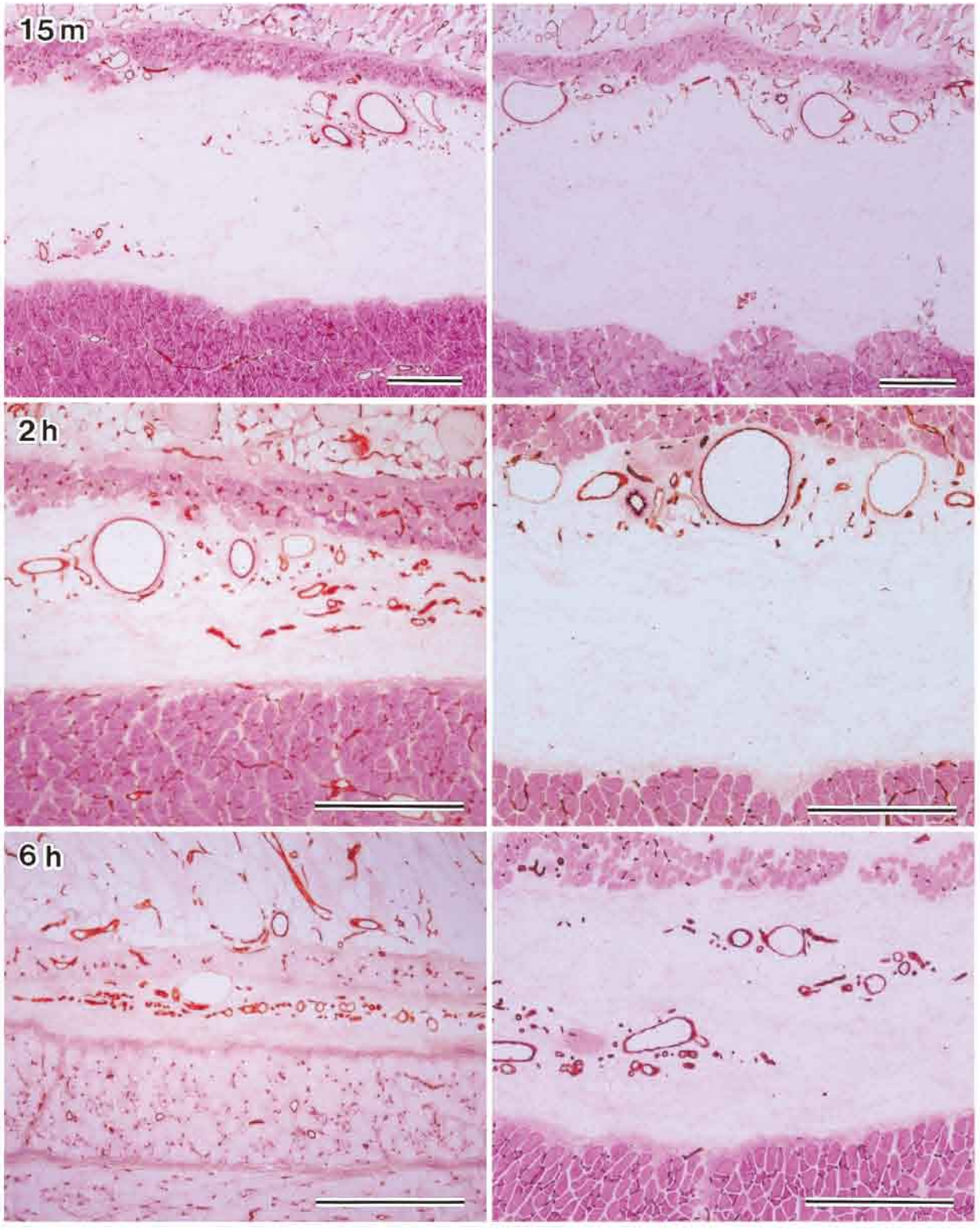


Fig. 8. Total cross sectional area of blood and lymphatic vessels pre- and post-injection. Bars indicate the cross sectional area of blood and lymphatic vessels. Empty portions indicate lymphatic vessels (n = 3). *: $P<0.05$ vs pre-injection



Figs. 9. and **10.** Legends on the next page.

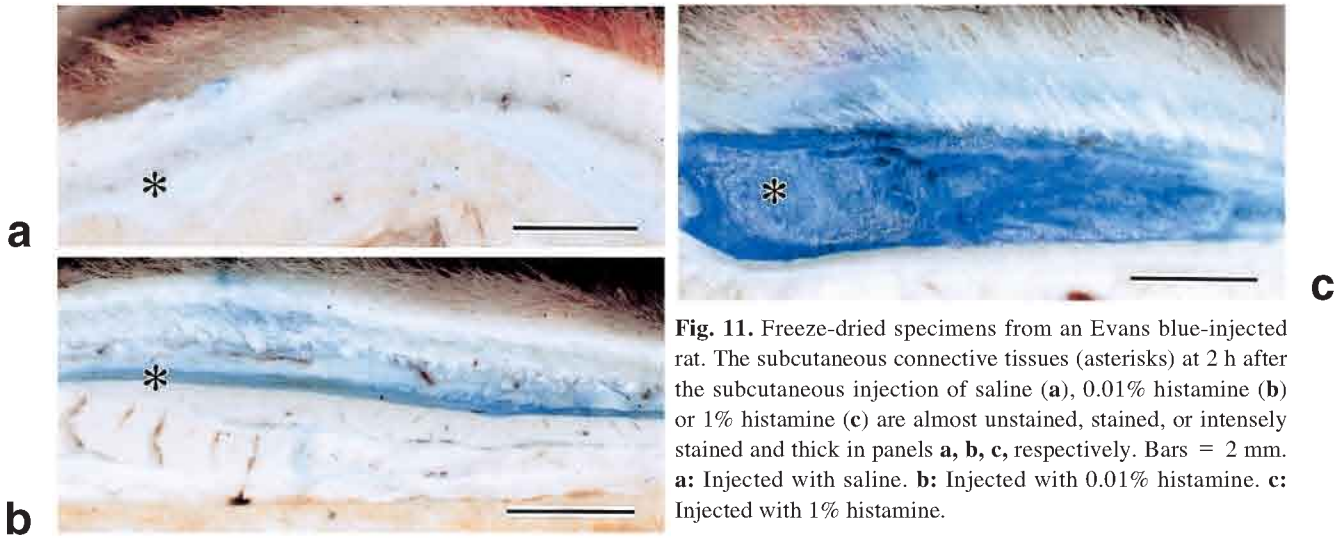


Fig. 11. Freeze-dried specimens from an Evans blue-injected rat. The subcutaneous connective tissues (asterisks) at 2 h after the subcutaneous injection of saline (**a**), 0.01% histamine (**b**) or 1% histamine (**c**) are almost unstained, stained, or intensely stained and thick in panels **a, b, c,** respectively. Bars = 2 mm. **a:** Injected with saline. **b:** Injected with 0.01% histamine. **c:** Injected with 1% histamine.

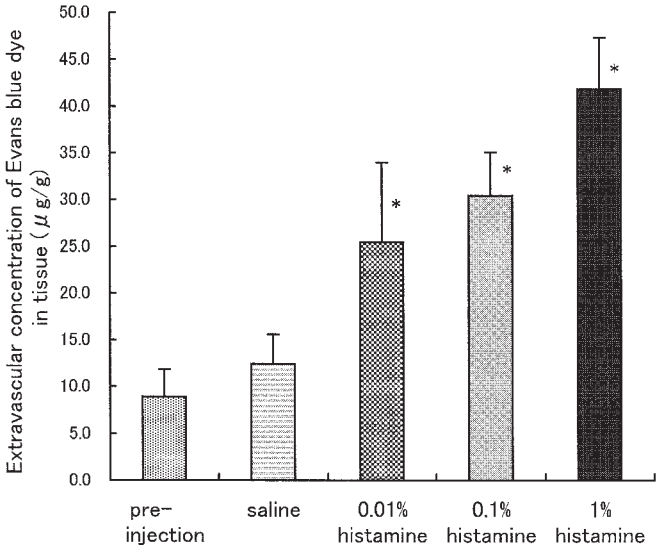


Fig. 12. Extravascular concentration of Evans blue dye in the tissue pre-injection and 2 h post-injection (n = 8). *: $P<0.05$ vs pre-injection.

Fig. 9. Sections of skin-muscle tissue after an injection of saline. Anti-PECAM-1 immunostaining. The magnifications in panels **b** and **c** are twice that in panel **a**. Bars = 500 μm. **a:** At 15 min post-injection. The subcutaneous connective tissue is remarkably thick. **b:** At 2 h post-injection. Numerous round blood and lymphatic vessels are seen. **c:** At 6 h post-injection.

Fig. 10. Sections of skin-muscle tissue after the injection of a 1% histamine solution. Anti-PECAM-1 immunostaining. The magnifications in panels **b** and **c** are twice that in panel **a**. Bars = 500 μm. **a:** At 15 min post-injection. The subcutaneous connective tissue is markedly thick. **b:** At 2 h post-injection. The subcutaneous connective tissue is thicker than that of the saline-injected counterpart (Fig. **9b**). **c:** At 6 h post-injection.

vessels (Wiig and Reed, 1981; Reed *et al.*, 1989). It is also considered that an increase in tissue fluid pressure causes intercellular junctions of the lymphatic endothelium to open, permitting the passage of fluid into the vessel (Pepper and Skobe, 2003). Drug solutions, either injected or leaked into the subcutaneous connective tissue for medical purposes, are highly likely to drain into blood and lymphatic vessels. Our observations may be helpful in treating and minimizing tissue damage in case of drug leakage.

Changes in blood and lymphatic vessels after saline injection

In the present study, the blood and lymphatic vessels — including small capillaries — were easily and reliably detected by immunohistochemistry. The total cross sectional area of the blood vessels was much greater than that of lymphatic vessels, while the extent of the increase was much larger in lymphatic than blood vessels (Fig.8). These findings can be explained by differences in number, shape, and distensibility between blood and lymphatic vessels. Blood vessels were numerous and generally round, whereas lymphatic vessels were small in number and compressed or collapsed (Schacht *et al.*, 2004) and more distensible due to lower intraluminar pressure than the veins (Ohhashi *et al.*, 1980). Lymphatic vessels can easily increase their cross sectional area by changing their compressed shape to become ovoid or round. In contrast, blood vessels are almost completely round and can only change slightly in shape after an injection. In addition, the increase in the number of blood and lymphatic vessels was only minimal (1.1-fold) at 2 h after the saline injection when the total cross sectional area of the blood and lymphatic vessels was maximal (2.2-fold). These results indicate that the cross sectional area of each blood and lymphatic vessel increased 2-fold on average. Due to the scarcity of lymphatic vessels, this increase seems attributable to the dilatation of blood vessels. In fact, the cross sectional area of blood vessels alone, which can be calculated by subtracting the podoplanin-positive area from the PECAM-1-positive area, is much increased at 2 h compared with that before the injection (Fig. 8).

Histamine injection

Histamine is well known to cause vasodilatation (Owen *et al.*, 1980; Treede *et al.*, 1990; Bergh *et al.*, 1996; Evilevitch *et al*, 1999), increase vascular permeability, and produce edemas (Owen *et al.*, 1980; Mcleod *et al.*, 2005). It is established that transvascular leakage is mediated by histamine H_1- and H_3-receptors, and vasodilatation is mediated by histamine H_1-, H_2-, and H_3-receptors (Owen *et al.*, 1980; McLeod *et al.*, 2005). Therefore, increased transvascular permeability and — to at least some extent — vasodilatation are anticipated to occur simultaneously because these actions are mediated by the same receptors. In the present study, histamine clearly elicited a dose-dependent increase in the amount of Evans blue dye in the tissue, indicating an increase in transvascular permeability, while the vasodilator response was enhanced only at high histamine concentrations. In addition, at 2 h post-injection, the total cross sectional areas of blood and lymphatic vessels in all injected groups showed maximal values, i.e., approximately 2.2-fold regardless of the concentration of histamine. It is therefore conceivable that blood and lymphatic vessels dilate maximally at 2 h after the injection of saline alone. These findings suggest that the vasodilatation induced by a low histamine concentration is partly or totally masked by the effects of saline injection.

Reed *et al.* (1989) stated that the interstitial fluid volume is well regulated and reabsorption plays a very important role in this regulation. In steady-state conditions, it is generally believed that most tissue fluid returns to blood vessels, while 10-20% of tissue fluid returns to lymphatic vessels. In the present study, the drainage of tissue fluid apparently increased after the subcutaneous injection because the subcutaneous connective tissue decreased in thickness with time. Most of the fluid temporally stored within the tissue after the injection is presumably excreted into the blood and lymphatic vessels. The increase in cross sectional area of the blood and lymphatic vessels probably reflects the increased drainage of tissue fluid into these vessels. However, neither the precise mechanisms of vasodilatation and increased fluid influx into vessels — particularly after the injection of saline, nor the functional roles of collagen and elastic fibers in the regulation of tissue fluid volume were clarified in this study. Furthermore, it is unclear which portion of blood vessels was dilated for the absorption of tissue fluid. These problems remain for further investigations.

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