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Application of tomato lectin and phalloidin in morphological analyses of the vascular network of various tissues:
With special reference to postnatal development of skin

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Abstract

Only limited information is available on the microvasculature in postnatal development, because currently used methods are time-consuming. We report here on a method for easily observing the microvasculature. FITC-tomato lectin was injected into the systemic circulation of rats. After a specified period, the tissues were fixed and cut. Actin filaments were stained with Alexa Fluor® 594 phalloidin and the specimens were observed under a confocal laser scanning microscope. At 30 minutes after injection, vascular endothelia of the kidney and brain were stained with the lectin. Vascular smooth muscles were visualized by staining with Alexa-phalloidin. One hour after the injection, the lectin was washed from the vessels of the skin and kidney and excreted from the renal tubules. In contrast, the lectin remained in the brain for periods of up to 2 hours. In the postnatal development of skin, the microvasculature around hair follicles and subepidermal regions increase in number within the first week, accompanied by the development of hair follicles. The fluorescent intensity of the vasculature of muscle flaps was too weak to permit analysis. The use of confocal microscopy and tomato lectin is useful for visualizing the microvasculature in various tissues/organs.

Key words *: tomato lectin, phalloidin, microvasculature, postnatal development of skin, confocal microscopy*

I. Introduction

The microvasculature, which is composed of arterioles, the capillary bed and venules, plays a fundamental role in physiological responses and pathological conditions (e.g., inflammation, tumors) of tissues/organs. In transplantation or reconstruction, the blood circulation in tissues/organs needs to be considered. For example, in reconstructive surgery, partial necrosis of the muscle flap probably occurs due to microcirculation failure. However, at present, details of the microvasculature in all tissues/organs are not well understood.

Radioangiography can be used to observe large vessels, but the microvasculature cannot be clearly observed. It is difficult to observe the entire arborization of the microvasculature in conventional tissue sections, because the vessels are thin and run in various directions. Three-dimensional reconstruction by consecutive

serial sections is time-consuming work[1]. In other methods, a resin cast of vessels under scanning electron microscopy shows only the vascular configuration, without providing information on the characteristics of vessels (e.g., the presence of smooth muscles), and the positioning of vessels in the tissues is unclear[2,3].

Confocal laser-scanning microscopy has recently been developed, and its optical slicing is capable of providing clear fluorescent images of thick sections. Three-dimensional reconstruction by confocal microscopy is both rapid and easy. Therefore, if there is a suitable florescent dye for staining the vascular components, it would facilitate the study of the vascular configurations in tissues/organs.

Lectins are nonimmunoglobulin glycoproteins that recognize and bind to specific sugar moieties on the cell membrane. They are involved in a variety of biological processes, such as cell-cell and host pathogen interactions and innate immune responses[4]. Tomato lectin (from *Lycopersicon esculentum*) is a very stable glycoprotein containing about 50 percent arabinose and galactose[5,6]. This lectin is composed of a single polypeptide of about 100,000 daltons that may form an aggregate in solution. Like other lectins that bind N-acetylglucosamine oligomers, tomato lectin prefers trimers and tetramers of this sugar[5]. One of the characteristics of this lectin is that it binds to endothelial cells without any species differences. Hence, tomato lectin has been applied to the staining of vessels[7-12]. However there is only one report of the staining of vascular networks with tomato lectin and confocal microscopy observations, to reconstruct the microvasculature of tissues/organs[11].

The aim of the present study was to determine whether tomato lectin staining can be used to visualize the microvasculature of various tissues/organs, and whether such staining can be applied to the reconstruction of the vasculature using confocal microscopy. To this end, we observed the postnatal development of the microvasculature of skin, because information on the microvasculature of neonate skin is limited, only changes in the microvasculature in young adult animals having been studied[13,14]. We also examined whether the staining method could be used in an evaluation of the blood stream of a muscle flap.

II. Materials and Methods

1. Animals

Rats and rabbits were used in the present study. Experiments were conducted in a manner consistent with the guidelines of the ethics committee for animal treatment of Iwate Medical University. Adult male (12 weeks-old) and pregnant (18th day of gestation) Wistar rats were purchased from SLC (Shizuoka, Japan). Neonates that gave birth from pregnant rats were also used in this study. They were kept in cages in the animal laboratories for medical research of Iwate Medical University, under constant conditions (temperature 20-24°C, relative humidity 53-57%, and LD 12:12 with light from 08:00 to 20:00). They were allowed free access to drinking water and standard laboratory food. Male rabbits (weighting 2.0-2.4 kg were

purchased from the Oriental Yeast Co. (Tokyo, Japan) and were also housed in single cages at a relative humidity of 73-77% and a temperature between 12 and 17°C.

2. Staining of blood vessels of rat tissue

To stain the endothelia of blood vessels, we used a lectin from the tomato (*Lycopfluoersicon esculentum*) [7-12]. We injected fluorescein (FITC) labeled tomato lectin (FL-1171, Vector Laboratories, Burlingame, CA, USA) into the blood stream. In adult rats, 200, 400, or 500 μg/ml of FITC-labeled lectin was injected via the tail vein using a small syringe with a 30G needle. The total injection volume was 0.05% of the body weight. After incubation for certain periods (30 minutes, 1 hour and 2 hours), the rats were killed by carbon dioxide gas followed by perfusion via the left cardiac ventricle with Ringer's solution (147 mM NaCl, 4 mM KCl and 2.25 mM $CaCl_2$) at room temperature (17-25°C) using a peristaltic pump (about 4-8 ml/min). In the case of neonates, we injected the lectin (500 μg/ml, total injection volume of 0.05% of body weight) via the jugular vein. After incubation for certain periods (5, 10, 30 minutes and 1 hour), they were killed and perfused, as described above.

The animals were perfused with a fixative containing 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) at room temperature. The brain, kidneys and skin from the abdominal area and dorsal forefoot were collected and immersed in the same fixative for 2 days.

3. Observation of the vascular structures of tissue specimens

The fixed specimens were then embedded in 5% low-melting-temperature agarose (Bio-Rad Laboratories, Hercules, CA, USA) and cut using a microslicer (Leica VT1000S, Leica, Göttingen, Germany) in 200 μm thick sections. Actin filaments were stained with Alexa Fluor® 594 phalloidin (2 units/10 μl methanol in a stock solution: Invitrogen-Molecular Probes, Eugene, OR, USA), to observe other structures of the blood vessels (i.e., smooth muscles). They were then embedded in an aqueous mounting medium (Aqua Poly/Mount, Polyscience, Warrington, PA, USA).

The specimens were observed under a confocal microscope (LSM 510, Zeiss, Jena, Germany). It is possible that some cross-talk of the fluorescent dyes could occur in multicolor applications, because the spectrum of the beam-splitter of the microscope is incomplete. Therefore, the multi-track scanning mode was applied during image acquisition. The vascular network of the skin specimens was reconstructed three-dimensionally using a computer assisted image analysis system (LSM Image Examiner, Zeiss, Jena, Germany).

4. Observation of blood vessels of a skin flap perfused with tomato lectin

We evaluated whether the lectin-perfusion method was capable of estimating blood circulation in a tissue (e.g., flap microcirculation). To this end, muscle flaps were injected with tomato lectin via an artery. A male rabbit was anesthetized intravenously with pentobarbital sodium (40 mg/kg, diluted in 0.9% NaCl saline) and the latissimus dorsi muscle flap was raised by the usual method [15]. This muscle flap is one of the flaps commonly used for the reconstruction of defects in

the body surface.

The fluorescent dye labeled tomato lectin (1 mg/ml; TL-1176, Vector) was then injected (1 ml) via an axillary artery using a small syringe with a 30G needle. Because a longer wave length of excitation and emission light can penetrate deeper into tissue, Texas Red-labeled lectin was used, instead of the FITC-labeled material. Fifteen minutes after the injection, the vasculature in the flap was observed under a fluorescence stereomicroscope (SZX12, Olympus, Tokyo, Japan) or a confocal microscope.

III. Results

1. Cerebral vasculature

We examined whether the endothelia and smooth muscles were stained with FITC-labeled tomato lectin and Alexa Fluor® 594 phalloidin, respectively, in an attempt to distinguish between arterioles and capillaries. To this end, we observed the vascular networks of the cerebral cortex, because other connective tissue elements and native autofluorescent components were minimal.

The double fluorescent staining permitted the endothelia and vascular smooth muscles of subarachnoid arterioles to be clearly observed (Figs. 1, 2 A-C). No cross-talk of the fluorescent dyes was observed when the multi-track mode was used in the microscopy (Fig. 1). The Alexa Fluor® 594 phalloidin staining of smooth muscles was often faint in intracerebral vessels (Figs. 2 A-C). In the blood stream, the lectin bound only to the surface of the endothelia, although, in a preliminary examination, when the tissue section was immersed in a solution containing the lectin (200 μg/ml), other structures (e.g., collagen fibers) were also stained. In the perfusion method, an injection of 200 μg/ml of lectin was not sufficient to stain the endothelia, but after an injection of 400 or 500 μg/ml, fluorescence was clearly detected.

The arborization of an artery in the brain was characteristic. Small arterioles (< ca. 50 μm in diameter) branched out from arteries (ca. 200-400 μm in diameter) or large arterioles (ca. 70-100 μm in diameter) at an obtuse angle in the subarachnoid space. The branched arterioles ran in the subarachnoid space for less than a few millimeters, passed through the pia mater, and then entered the cerebral cortex perpendicularly. Many capillaries then perpendicularly branched out in the cerebral cortex (Fig. 2 A).

2. Incubation time for staining the vasculature

To determine the optimal incubation time for staining the endothelia of vessels in various tissues (brain, kidney and skin), rats were allowed to rest for certain periods (5 minutes, 30 minutes, 1 hour and 2 hours) after the injection of the tomato lectin (Fig. 2). In the brain, 5 minutes after the injection, the staining was faint. The vasculature was most clearly depicted 30 minutes after the injection; the endothelia of most vessels, including capillary networks, could be visualized (Fig. 2 A). One hour after the injection, the fluorescent intensity of brain vessels was decreased slightly and not all the vessels were stained (Fig. 2B). Two hours after the injection, detectable

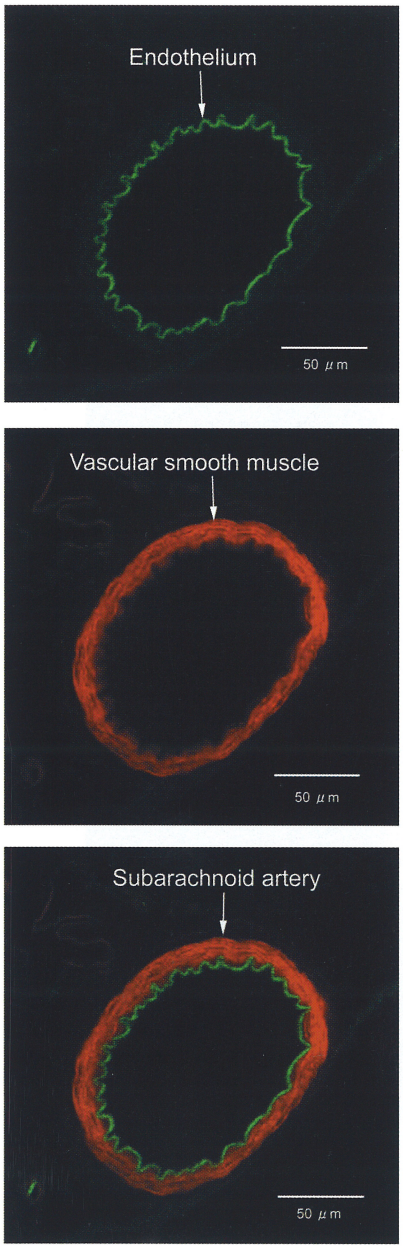


Fig. 1. Confocal images of a subarachnoid artery. FITC-labeled tomato lectin (green peudocolar), Alexa Fluor® 594 phalloidin (red pseudocolar) staining.
Upper: Endothelia stained by the FITC-labeled tomato lectin. Middle: vascular smooth muscle cells stained by Alexa Fluor® 594 phalloidin. Lower: merged image, indicating no cross-talk of fluorescence. Bar 50 μm.

levels of fluorescent lectin still remained (Fig. 2C).

In the kidney, within 5 minutes after the lectin injection, the endothelia of the renal glomeruli and blood vessels around the renal tubules were stained, but those of relatively large vessels were not. Podocytes of the glomeruli, vascular smooth muscles and the brush borders of renal tubules could be visualized with Alexa-phalloidin (Fig. 2D). Thirty minutes or 1 hour after the injection, the endothelia of the renal glomeruli were fragmentally stained with the lectin, and the fluorescence of the peritubular blood vessel endothelia had decreased. On the other hand, epithelial cells of convoluted tubules of the cortex and endothelia of arteries were stained (Fig. 2E). Two hours after the injection, the fluorescent intensity of the endothelia of the renal glomeruli and peritubular vessels had decreased gradually, whereas the endothelia of large arteries remained stained. Epithelial cells of convoluted renal tubules in the cortex fluoresced intensely, but not those in the medulla (Fig. 2F).

In the skin of adult rats, the incubation time required to adequately stain the endothelia of vessels was 30 min, while it was 5 minutes in the case of neonates. The sebaceous glands were also readily stained (Fig. 3). One or 2 hours after the injection, the staining of vessels had decreased considerably, while the sebaceous glands of skin specimens fluoresced.

These findings indicate that the affinity of tomato lectin to the endothelia of renal capillaries and neonate vessels was weak and that the dye was easily washed out.

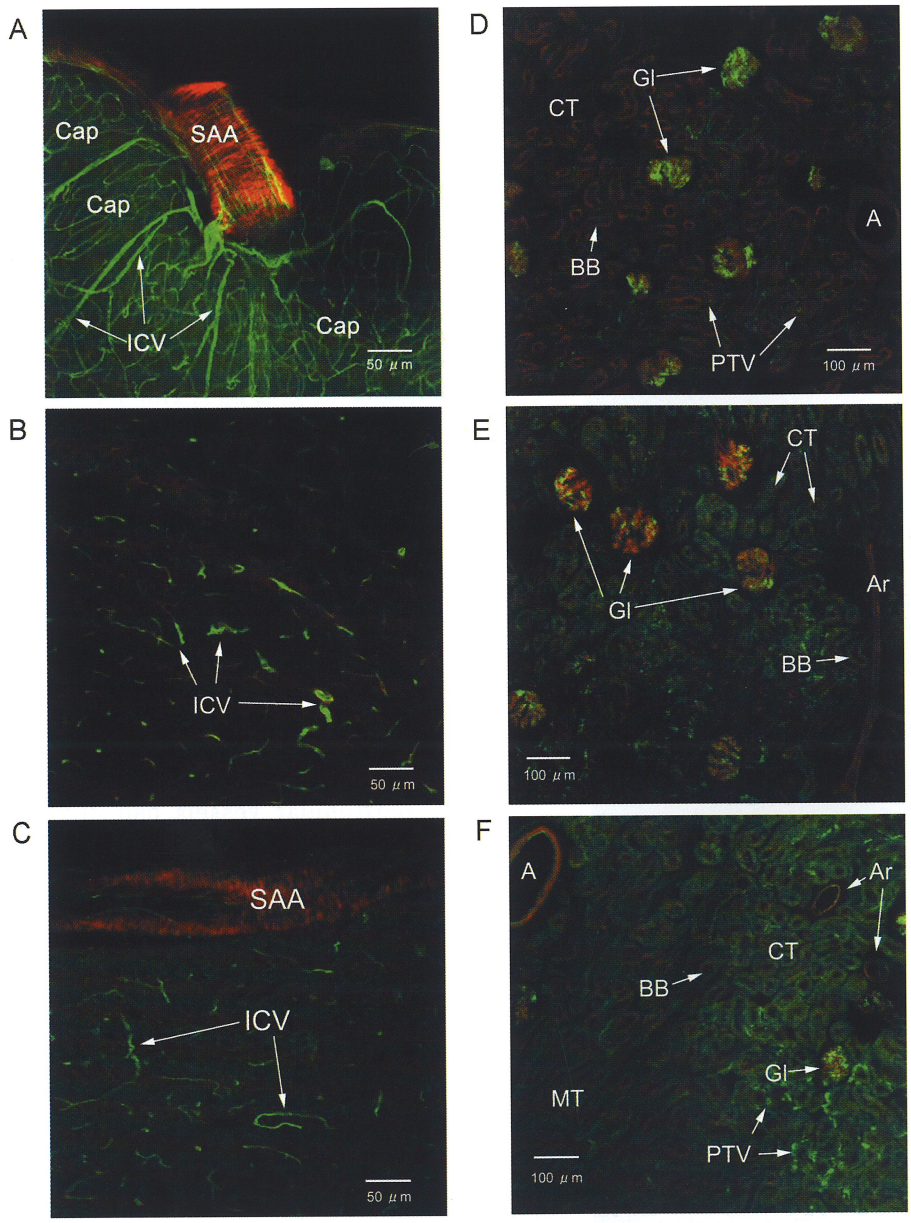


Fig. 2. Confocal images showing double staining by FITC-labeled tomato lectin (green) and Alexa Fluor® 594 phalloidin (red) in brain (A-C) and kidney slices (D-F).

A: Thirty minutes after the injection in the brain. SAA: subarachnoid artery, ICV: intracerebral vessels, Cap: capillaries.

B: One hour after injection.

C: Two hours after injection.

D: Five minutes after injection in the kidney. A: arteries, BB: brush borders, CT: cortical renal tubules, Gl: renal glomeruli, PTV: peritubular vessels.

E: One hour after injection. Ar: arterioles.

F: Two hours after injection in the area of the cortico-medullary junction. MT: medullary renal tubules. Bar 100 μm.

The lectin, however, was found to bind more strongly to vascular endothelial cells of the brain, compared to other organs.

Variations in the affinity of the endothelia with the lectin in various compartments should be considered in morphological analyses using this lectin.

3. Postnatal development of the vascular network in skin

Using an adequate concentration of lectin (500 μg/ml) and an appropriate incubation time (30 minutes for adults, 5 minutes for neonates), the postnatal development of the tissue circulation of the skin was examined. In addition, the microvasculature of the skin was three-dimensionally reconstructed from consecutive optical slice images.

The vascular network of a 0 day-old rat skin was sparse (Fig. 3 A). The vessels were sinusoidal; their luminal diameters were irregular (ca. 2-8 μm in diameter). Sinusoidal vessels just below the epidermis ran horizontally. The epidermis, hair follicles and dermal skeletal muscles were stained with Alexa phalloidin. The smooth muscles of subepidermal vessels were faint, and only a few arteriole smooth muscles of the deep dermis could be clearly identified. The hair follicles were very small and it was difficult to identify the hair structure.

In 2 day-old rats, the vascular network of the skin had developed (Fig. 3 B). The diameters of the vessels were relative constant (ca. 8 μm in diameter), compared to those in the 0-day rat skin. The vessels in the dermal papilla rose perpendicularly from subepidermal vessels, running horizontally. These subepidermal vessels showed few smooth muscles. In the deep dermis, arterioles were evident (ca. 50 μm in diameter) and venules (ca. 40 μm in diameter) possessing smooth muscle layers were observed. The microvasculature (ca. 8 μm in diameter) of the dermal muscle could be clearly observed, and ran tortuously.

In 4 day-old rats, networks of subepidermal vessels had developed (ca. 10 μm in diameter), and some possessed smooth muscles (Fig. 3 C), indicating that these vessels were terminal arterioles. The sebaceous glands were developed, and were stained with the lectin. In this period, the hair follicles became large, but few hairs showing auto-fluorescence were recognizable.

In 8 day-old rats, the microvasculature network developed around large hair follicles, although the staining was weak. In hair follicles, the medulla and sheath of hairs were distinguishable (Fig. 3 D). Sebaceous glands stained with the lectin were also developed. Because the number and size of hair follicles increased drastically, it was difficult to obtain an overview of the subepidermal vasculature. At higher magnification, they consisted of terminal arterioles which possessed a thin smooth muscle layer. The vessels of the dermal muscles increased in number and density.

In adult rats, vessels between hair follicles were observed at lower magnification (Fig. 4 A). They possessed evident smooth muscles, indicating that these were arterioles. The hair showed a green-autofluorescence under blue-laser excitation. Around the hair follicles, dense vascular networks (ca. 5-15 μm in diameter) could be observed

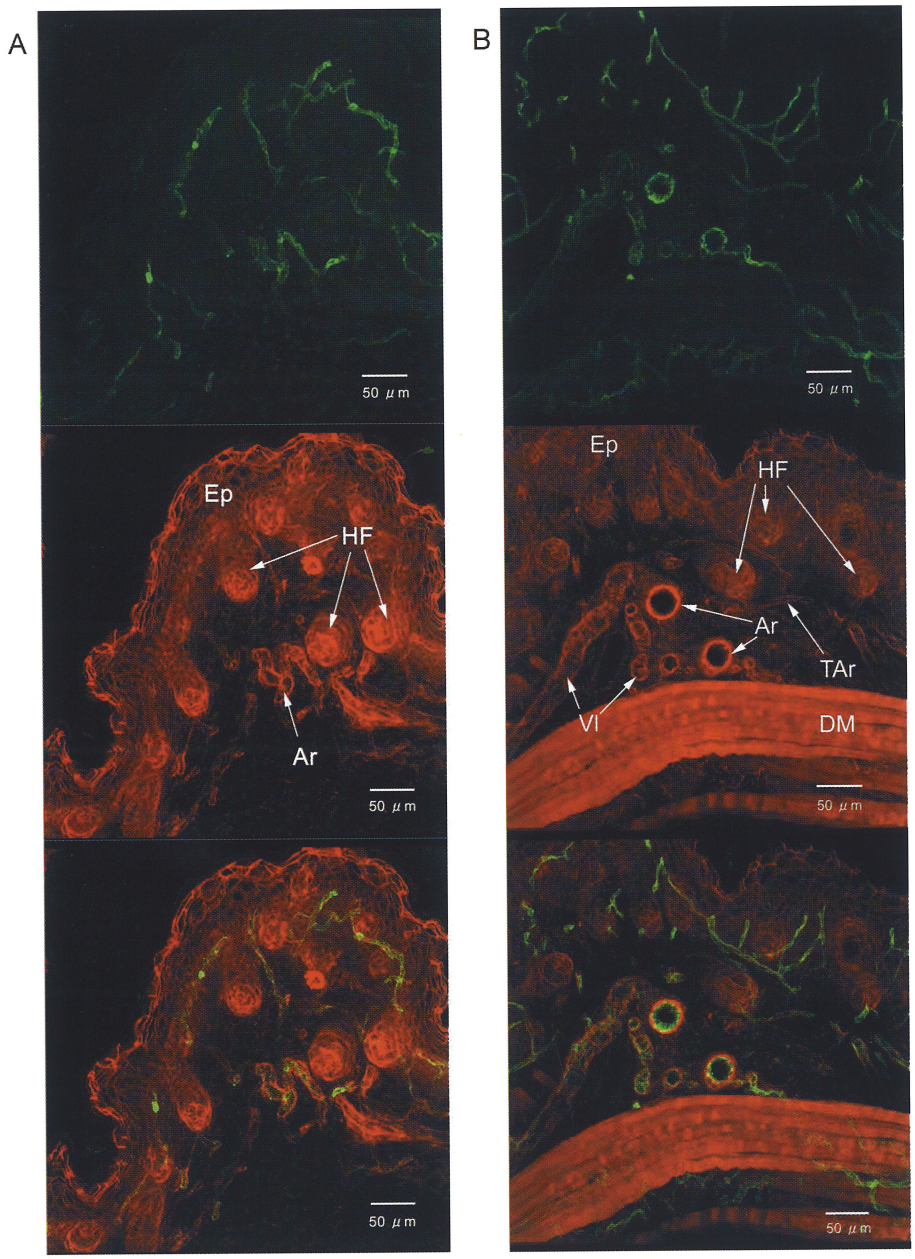
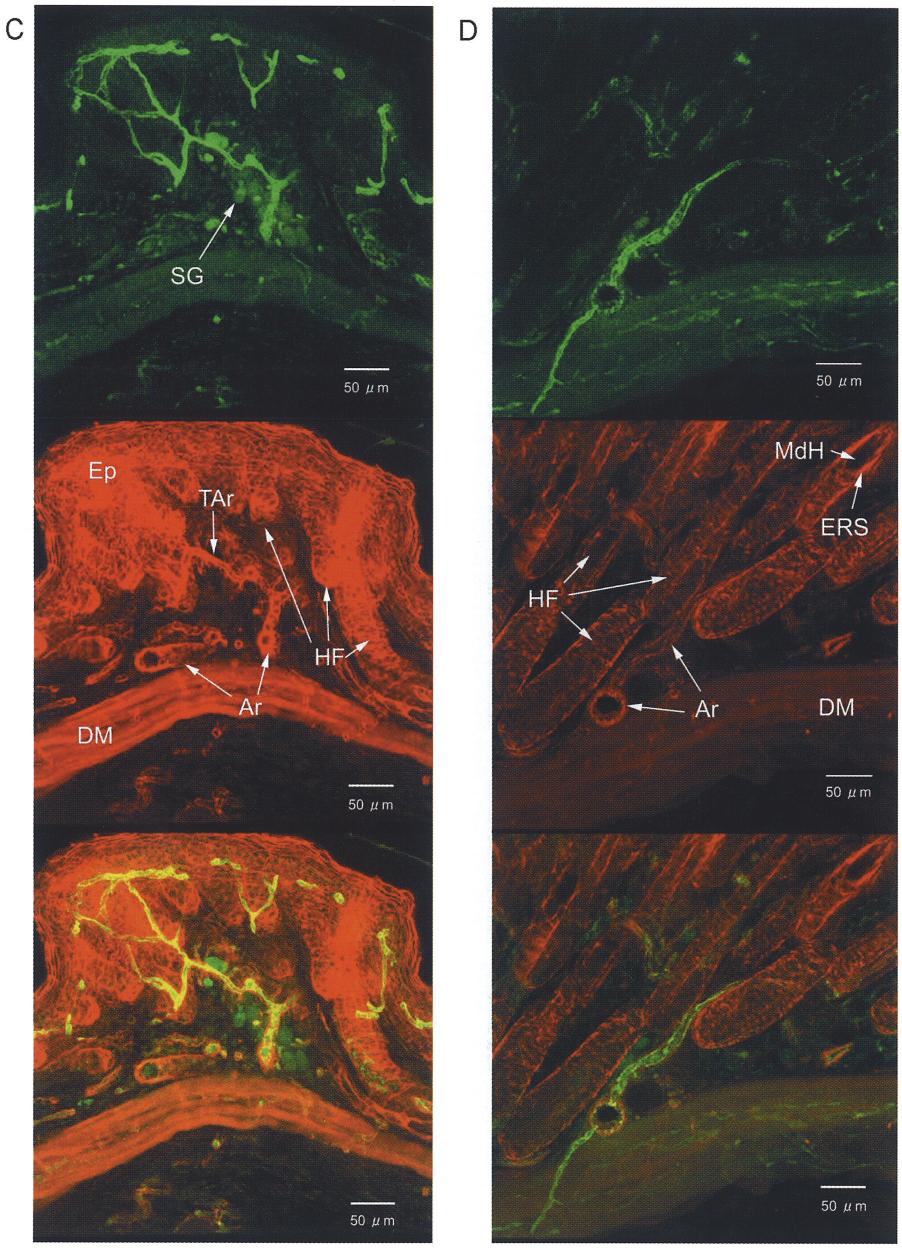


Fig. 3. Confocal images of the skin microvasculature of neonatal rats (0-8 days). Upper: FITC-tomato lectin image, middle: Alexa Fluor® 594 phalloidin image, lower: merged image. The endothelia were stained by the FITC-tomato lectin (green). Epidermis (Ep), hair follicles (HF), vascular smooth muscles and dermal muscles (DM) were stained by Alexa Fluor® 594 phalloidin (red).
A: Abdominal skin of a 0 day-old rat. Ar: arterioles
B: Abdominal skin of a 2 day-old rat. Vl: venules
C: Abdominal skin of a 4 day-old rat. TAr: terminal arterioles, SG: sebaceous glands
D: Abdominal skin of an 8 day-old rat. ERS: external root sheath, MdH: medulla of hair. Bar 50 μm.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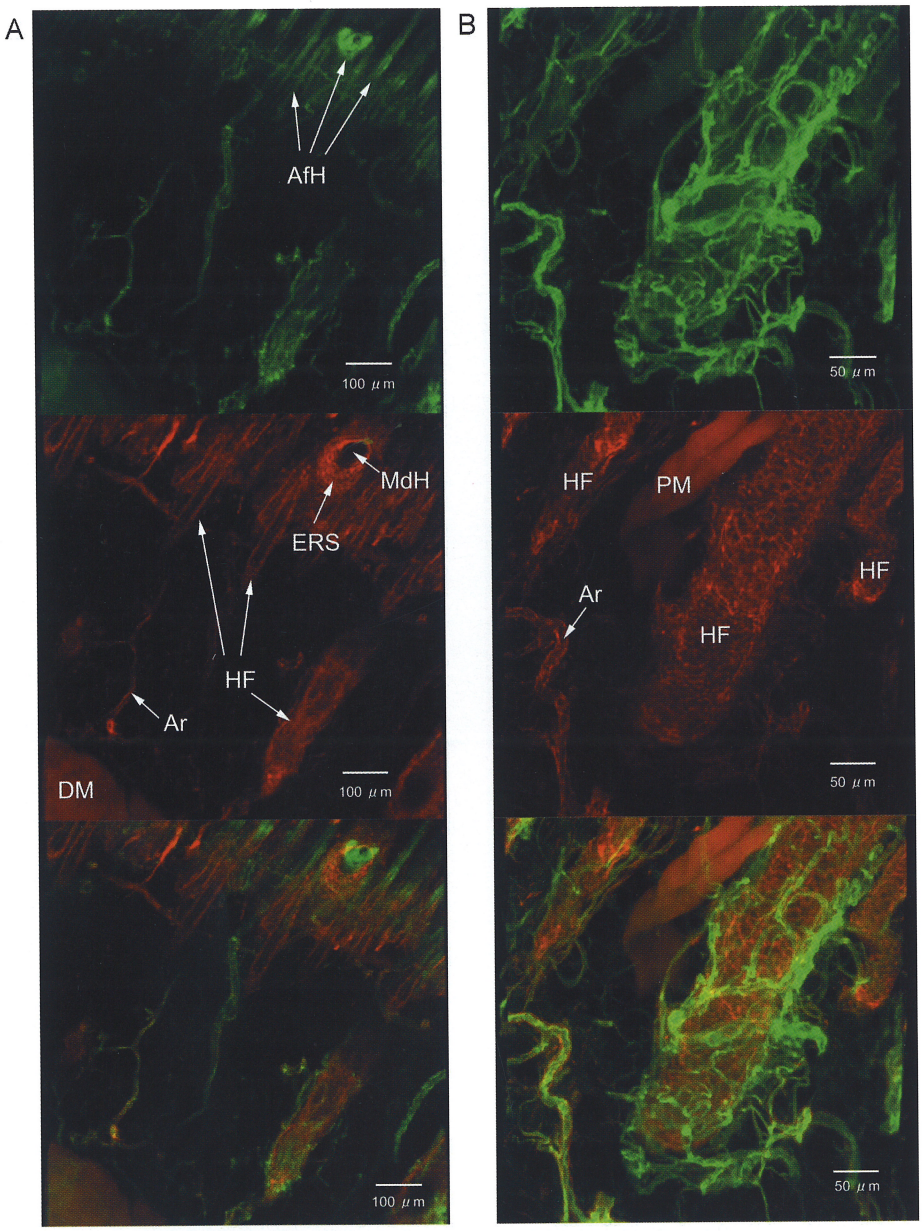

Fig. 4. Confocal images of the skin microvasculature of an adult rat. Upper: FITC-tomato lectin image, middle: Alexa Fluor® 594 phalloidin image, lower: a merged image. Endothelia were stained by FITC-tomato lectin (green). Epidermis (Ep), hair follicles (HF), vascular smooth muscles and dermal muscles (DM) were stained by Alexa Fluor® 594 phalloidin (red).

A: Low magnification images of the skin of an adult rat. Ar: arterioles, ERS: external root sheath, MdH: medulla of hair. Bar 100 μm.

B: High magnification images of hair follicles of the shallow layer of an adult rat skin. PM: pilomotor muscle. Bar 50 μm.

(Fig. 4 B). The double staining method using the FITC-labeled tomato lectin and Alexa phalloidin was useful for distinguishing between small arterioles and wide capillaries. The vascular network around the follicle lacked a smooth muscle layer, suggesting that the network contained sinusoidal capillaries. On the other hand, the vessels between hair follicles (ca. 15 μm in diameter) clearly showed smooth muscles that were stained with Alexa phalloidin, indicating that these were terminal arterioles. Pilomotor muscles could be seen beside hair follicles. There was no significant difference between skins of the abdominal area and dorsal forefoot.

4. Staining the vasculature of the muscle flap

To determine whether vital staining with the tomato lectin was capable of evaluating the tissue circulation of a graft, we injected the lectin into the vessel of the latissimus dorsi muscle flap of a rabbit. The flap was placed under a fluorescent stereomicroscope and observed in a dark room; the Texas Red fluorescence of vessels was detected (data not shown), but a quantitative evaluation of tissue circulation was not possible. The flap on the cover slip was then examined under a confocal laser scanning microscope, but the fluorescent intensity was too weak to permit the vascular network to be visualized.

IV. Discussion

Previous studies using the vital staining of vessels with tomato lectin attempted to examine capillary formation from exiting vessels (i.e., angiogenesis) in inflammatory lesions or tumors[8, 16, 17]. The present study revealed that lectin staining *via* the blood stream can be useful for analyzing the structural characteristics of the normal microvasculature (arterioles and capillary bed). In addition to the staining of endothelia, the visualization of smooth muscles by fluorescent phalloidin was capable of distinguishing between terminal arterioles and capillaries.

1. Comparison of previous methods of observing vascular networks

Two methods are typically used to observe the microvasculature in various organs and tissues. The first is the casting method; Resins are injected into the vessels, the cellular and tissue components removed by digestion, and the resin cast of the vessels is then observed under a scanning electron microscope[2, 3]. The second is a tracing method: Outlines of the microvasculature of light or electron micrographs of consecutive serial sections are depicted, the outlines are superimposed, and three-dimensional images are then reconstructed[1]. However, Resin cast images show only the vascular network, and it is difficult to depict the relationship between vessels and other tissue elements (e.g., parenchyma cells or interstitial connective tissues). In the case of reconstruction based on serial images, the distortion of sections is inevitable, and it is difficult to set appropriate landmarks on the accumulated outlines. In addition, these methods require skilful techniques for the injection of viscous resins and cutting the serial sections. It frequently requires over 1 week to obtain

an image after tissue sampling. If one wished to compare the microvasculature of different tissues/organs or examine various experimental conditions, the previous methods are clearly not suitable, because of the time and skill involved.

The use of immuno-fluorescent-histochemistry in the study of vascular components is a currently-used method for observing the microvasculature of tissues/organs. In a preliminary examination, the staining of the endothelium by an anti PECAM-1 (platelet/endothelial cell adhesion molecule-1) antibody[18, 19] was attempted. Staining of the vasculature of neonates was unsuccessful, although the vessels of adults were stained. It is possible that there may be species differences in immunohistochemistry.

In contrast to the methods described above, the intravital perfusion method using the tomato lectin and reconstruction by confocal microscopy is easy and speedy, providing three-dimensional images of the tissue of interest within 3 days. The tomato lectin binds to the endothelia of all species, i.e., there are few species differences[8]. The original tomato lectin perfusion method targeted capillaries, and in the present study the method was refined to visualize other factors (e.g., vascular smooth muscles, hair follicles and so on). The shortcoming of the lectin perfusion method is as follows: Non-perfused vessels in vasculogenesis could not be stained by the lectin. However, this feature may be useful in evaluating haemodynamics in the vessels.

2. Postnatal development of blood vessels in the skin

Whereas many studies have focused on the mechanisms of angiogenesis in pathological skin tissue, vasculogenesis or angiogenesis in normal development has received comparatively less attention. The present method for visualizing the microvasculature was used to observe the postnatal development of skin.

After birth, the structure of the skin of the rat undergoes drastic changes within one week, especially the hairs and hair follicles. Neonatal rats are born without body hair, and only small hair follicles are usually observed. The microvasculature network in the subepidermal regions was loose. Accompanying the development of hair follicles, the subepidermal microvasculature increased in number and the smooth muscle layer appeared in the vessels. It is well known that hair follicles are surrounded by capillary networks[2]. The present study revealed that the microvasculature around hair follicles was sinusoidal capillaries, and these appeared at around one week, in the period when body hairs began to grow. In contrast to the drastic change in the microvasculature of the subepidermal regions, the microvasculature of dermal skeletal muscle had already developed within 2 days. In accordance with this, few changes in the relative large vasculature in deep regions during aging have been reported, although the microvasculature around hair follicles was not mentioned[20].

3. Heterogeneity of the endothelia

Lectins are ligands that bind to various carbohydrates, which are essential factors of cell membranes, including the luminal surface of endothelium[4, 8]. In the

present study, the adequate incubation time for the staining of endothelia differed in postnatal development, and in various tissues/organs and in the size of the vessels. The speed of luminal perfusion in vessels might affect the differences in staining. Generally, the endothelia of different tissues/organs possess specific adhesion molecules (most of which are carbohydrates of the cell membrane). Therefore, it is more likely that the affinity and endocytosis of the tomato lectin for the endothelia in various vessels is uneven, although the lectin is able to bind to all endothelia[8]. The reason why the fluorescence of lectin remains on endothelia for a long time is that lectin is probably ingested from the luminal surface of endothelia into the endothelial cells.

4. Excretion of tomato lectin from the body

In the present study, the application of a vascular perfusion method using tomato lectin to evaluate the circulation in human tissues was also an aim. If one could introduce a material into clinical medicine, the excretion dynamics would need to be examined. However, no reports on the excretion of tomato lectin are available, although the lectin has been studied as a putative non-toxic lectin with the potential for drug targeting/delivery[21-24].

The staining of renal tubules by tomato lectin indicates that the lectin is secreted *via* the tubules to the urine, and some exocrine cells may play a role in excretion of the lectin. Therefore, the tomato lectin could induce functional disorders in renal tubules. The binding of tomato lectin to the endothelia may transiently interfere with the functions of the luminal cell membrane.

5. Prospects for applications of tomato lectin

Other methods are available for evaluating the circulation in tissues/organs: 1) FITC-dextran was injected into vessels and the florescence was measured by means of a fluorescent microscope[25,26], 2) indocyanin-green was used as an injection-dye and tissue perfusion was evaluated by laser video angiography[27-32]. These techniques have been applied clinically to evaluate tissue circulation. Tomato lectin was used as an intravital perfusion method in human tissues[9].

We obtained clear images by the vascular perfusion of tomato lectin in tissues of experimental animals, therefore we expected that the present method would have the potential for evaluating the vascular perfusion of the muscle flap. However, the attempt failed. Before clinical applications of the lectin, there are many factors (e.g., penetration of excitation and emission light, adequate imaging system, examination of toxicity and determination of the excretion mechanism) that need to be solved.

In conclusion, the vascular imaging by tomato lectin perfusion and phalloidin staining described here will be convenient for observing the microvasculature of various tissues/organs in physiological or pathological conditions. In the near future, to estimate dynamic changes in the binding ability of endothelia of various vessels, we plan to measure the time course for the fluorescent intensity of the lectin when it is bound to the endothelia.

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