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A long-distance fluid transport pathway within fibrous connective tissues in patients with ankle edema

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Abstract.
OBJECTIVES: Although the microcirculatory dysfunctions of edema formation are well documented, the draining pattern of dermal edema lacks information. This study was to assess the potential drainage pathways of the interstitial fluid in patients with ankle edema using the anatomical and histological methods.
METHODS AND RESULTS: Four amputees of lower leg participated in this study. Fluorescent imaging agent was injected into lateral ankle dermis in one volunteered patient before the amputation and three lower legs after the amputation. Physiologically in the volunteer or enhanced by cyclical compression on three amputated limbs, several fluorescent longitudinal pathways from ankle dermis to the broken end of the amputated legs were subsequently visualized and studied using histological methods, laser confocal microscopy and electron microscopy methods respectively. Interestingly, the fluorescent pathways confirmed to be fibrous connective tissues and the presence of two types: those of the cutaneous pathway (located in dermis or the interlobular septum among adipose tissues within the hypodermis) and those of the perivascular pathway (located in connective tissues surrounding the veins and the arteries). The intrinsic three-dimensional architecture of each fluorescent pathway was the longitudinally running and interconnected fibril bundles, upon which, an interfacial transport pathway within connective tissues was visualized by fluorescein.
CONCLUSIONS: The current anatomical data suggested that a unique long-distance transport pathway composed of oriented fibrous connective tissues might play a pathophysiological role in draining dermal edema besides vascular circulations and provide novel understandings of general fibrous connective tissues in life science.

Keywords: Connective tissues, vascular circulations, interstitial fluid

1. Introduction

Skin edema in the distal extremities is a commonly observed feature in patients with diabetic foot, arteriosclerosis obliterans, consequence of heart failure, varicose veins, lymphedema, myxedema and etc., which represent the imbalance of fluid exchange across the capillary membrane in skin and might lead to a change from the malfunctions of skin to severe diseases like foot ulceration and even

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amputation of lower leg. Several predisposing factors have been proposed, such as atherosclerotic occlusion, loss of arteriolar pre-capillary vasoconstriction and impaired sympathetic veno-arteriolar axon reflex resulting from diabetic neuropathy and capillary permeability and vascular leakage due to atherosclerotic and microcirculatory endothelial dysfunctions [1, 16, 17]. However, few studies focused on the perihemorheology of fluid and structures in the perivascular or interstitial spaces, such as loose connective tissues.

Recently, increasing experimental data suggest a phenomenon of fluid flow through perivenous loose connective tissues (PLCTs) found in rabbit and mice, contributing to the drainage of fluid from the superficial layer of extremities into the pericardial cavity or the clearance of interstitial solutes in the brain or the "perivascular-spaces-like" pathways beside lymphatic and blood vessels under the skin of extremities or in the thymus, lungs and even in tumours [8, 9, 12, 14]. In healthy humans, it was also found that longitudinal non-vascular transport pathways were surrounding the venous vessels or within the subcutaneous tissues in limbs by the hypodermic injection of paramagnetic or fluorescent tracers into the skin [11, 13].

Herein, the present study aims to assess what the anatomical and histological structures of the longitudinal non-vascular transport pathways in limbs are and whether the loose connective tissues may involve in the removal of the interstitial fluid in humans. A point on lateral malleolus of ankle with "pitting edema" was chosen as a marker to explore the drainage pathways of skin edema using anatomical and histological methods in four amputated lower legs sampled from four patients who underwent an amputation surgery.

2. Materials and methods

2.1. The subjects involved

A total of four patients (September 2014 $\sim$ April 2015) with amputation of the lower leg were involved. One volunteered patient underwent severe foot gangrene due to arteriosclerosis obliterans in right lower leg, who was administered a hypodermic injection of imaging tracer before the amputation. The other 3 amputated lower legs, sampled from 3 patients with ankle edema and severe diabetic foot gangrene, were studied after the amputation. The research protocol was approved through the ethics committee of Beijing Hospital of the Ministry of Health (No. 2013BJYYEC-037-02). The decision for operation was obtained from the surgeon based on the condition of the patient and was not associated with the current scientific studies.

2.2. Method of visualizing the transport pathways in one volunteered patient undergoing the subcutaneous injection before the amputation

Before the amputation, one patient volunteered with signed informed consent to accept a hypodermic injection of fluorescein sodium solution (NaF) (0.3 mL, 0.5% diluted in physiological saline, Guangzhou Baiyunshan Mingxing Pharmaceutical Co., Ltd), which was permitted for human use. The injection point was located in the middle of the shortest line between the apex of the lateral malleolus and the heel tendon of the lateral ankle and hypodermically injected by a 1-mL syringe at a depth of approximately 1-2 mm perpendicularly into the skin surface (Fig. 1). At 1 hour after the operation, the fluorescently stained tissues on the skin or within the subcutaneous tissues or the deeper tissues were recorded using a digital camera under blue-violet light. To visualize the fluorescent pathways, paralleling incisions at 5-cm intervals perpendicular to the long-axis of the lower leg were created in the skin from the injection point to the broken end and interesting tissues were sampled.

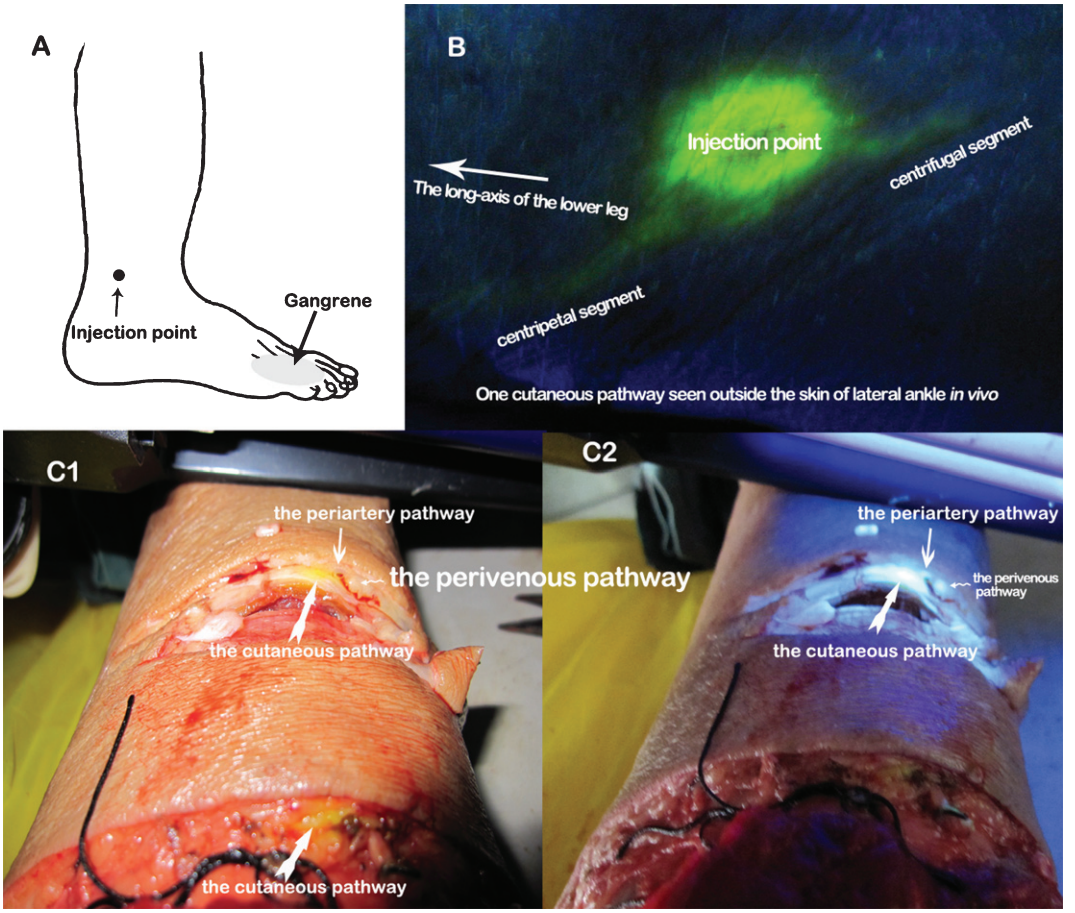

Fig. 1. Illustrations of the cutaneous and perivascular pathways in the amputated lower leg of the patient who received the injection before the amputation. **A**: the dark round dot was the injection point located on the shortest line between the apex of the lateral malleolus and heel tendon. The oval illustrated the position of the gangrene. **B**: under blue-violet lights, the centripetal and centrifugal segments of the fluorescent cutaneous path originating from the injection point were seen outside the skin of lateral ankle after the amputation. **C1**: stained by fluorescein, the cutaneous path under the skin, the periarterial path and perivenous path were seen under natural lights. **C2**: the same view of **C** under blue-violet lights. The cutaneous, periarterial and perivenous paths were highlighted.

2.3. Method of visualizing the transport pathways in the remaining 3 amputated lower legs by the mechanical manipulation

The remaining three patients did NOT receive hypodermic injection PRIOR TO the amputation. These individuals signed routine consent forms for the amputation operation and further pathological examinations and scientific examination of the amputated tissues. The dorsum of each amputated foot was selected as a control injection point and injected by 0.3 mL of the mixed solution, containing NaF (0.5% diluted in saline) and Rhodamine B (0.2% diluted in saline, Sigma-Aldrich, China). The amputated lower legs were refrigerated at 4°C for more than 2 hr of observation and subsequently used in the following studies. 2 hr later after the preservation, the same point located on the lateral malleolus was injected hypodermically before the following manipulation.

Repeated "cyclical compression" on the skin near the broken end of the amputated leg using a sphygmomanometer cuff was performed for approximately 90 min after the injection. The pressure was 50–60 mmHg, and the pressure-release frequency was 18–20 times per min.

2.4. Methods of histological study on the fluorescent pathways

After the amputation, frozen cross-sections of 5–8 μm in thickness were cut from the tissues containing the fluorescent pathways observed under a fluorescence microscopy. The same slides of the frozen sections and controlled samples were examined using Haematoxylin & Eosin (HE) staining and the combined staining methods of Van Gieson & Verhoeff Iron-Haematoxylin (VG+VIH), respectively.

The sections of the fluorescent pathways were immunohistochemically stained with antibodies dilution (1 : 200) against D2-40 (Dakocytomation, Glostrup, Denmark). The epitope retrieval was performed manually by using pressure cooker. Envision+HRP (Dakocytomation, Glostrup, Denmark) was subsequently used as secondary detection.

2.5. Method of two-photon laser scanning microscopy on the fluorescent pathways

The selected experimental tissues were the same samples as those of the frozen sections. Two-photon laser scanning microscopy (TPLSM, Olympus FV1000 and Spectra-Physics Mai Tai DeepSee) was used to study the experimental and controlled samples obtained from the skins, and the blood vessels including their surrounding connective tissues, as this imaging technique offered important advantages over confocal laser scanning microscopy, such as deep tissue penetration and high spatial resolution. TPLSM was performed at 960 nm for excitation and 495–630 nm for emission. For each sample, 25 slices were captured using a 0.1-μm interval at a 512 × 512 resolution.

2.6. Methods of SEM and TEM on the fluorescent pathways

The selected tissue samples prepared for SEM and TEM were the same as those obtained for the analysis of the frozen sections. These tissue samples were fixed overnight in 2.5 v/v% glutaraldehyde, followed by dehydration through graded solutions (30%, 50%, 70%, 90% and 100%; for 10 min each) of ethanol for SEM and acetone for TEM. For SEM, the samples were subsequently dried through critical point drying and imaged under ESEM (Quanta 200 FEG, FEI) in low vacuum mode. For TEM, the samples were embedded with resin after acetone replacement three times, and subsequently dried in an incubator under the following conditions: 30 °C for 12 h, 35 °C for 5 h, 37 °C for 12 h, 40 °C for 5 h, 45 °C for 12 h, 47 °C for 5 h, 50 °C for 5 h, 55 °C for 5 h, and 60 °C for 24 h. The samples were cut into 50-nm thick sections and imaged under TEM (H-7500, Hitachi).

3. Results

3.1. The fluorescent pathways in patient with the hypodermic injection before the amputation

In the volunteered patient who was hypodermically injected before the amputation by NaF, a single fluorescent pathway on the skin was observed in the amputated lower leg under blue-violet light (Fig. 1B). This fluorescently-stained cutaneous pathway originated from the injection point in the lateral ankle and travelled centripetally to the broken end of the leg. The cutaneous pathway was visualized outside the skin near ankle but invisible along the course of lower leg. When the deeper tissues of lower leg were exposed to open air, not only the cutaneous pathway under the skin, but also few fluorescently-stained perivascular pathways around blood vessels and fluorescently-stained nerves in the vicinity were stained by the fluorescein from ankle (Figs. 1C1-2, A, B). These fluorescent pathways were found from the injection point of ankle to the broken end of lower leg and travelled centrifugally for a distance of more than 10 cm.

3.2. The fluorescent pathways visualized in the remaining 3 amputated lower legs by the mechanical manipulation

The remaining three lower legs were injected by the combined imaging agents, NaF and Rhodamine B, AFTER the amputation surgery. On the dorsum of each amputated foot without the following manipulation, fluorescein was locally diffused around the control injection site after 2 hr of observation when the subcutaneous tissues were cut open.

The combined fluorescein injected into the point on the shortest line between the apex of the lateral malleolus and heel tendon (Fig. 1A) was driven centripetally by the repeated mechanical "cyclical compression" on the broken end of the amputated lower legs. After the tissues opened, the same fluorescent cutaneous pathway, fluorescent perivascular pathway and fluorescently-stained nerve can be found as well from the lateral ankle to the broken end of the amputated leg.

3.3. Histological structures of the fluorescent pathways

Fluorescence microscopy of the frozen cross-sections revealed that the anatomical structures of the fluorescent cutaneous pathways were located in the dermis (Fig. 2A1) or the interlobular septum among adipose tissues within the hypodermis (Fig. 2B1); the fluorescent perivascular pathways were in the fibrous loose connective tissues (including tunica adventitia) surrounding the veins (Fig. 2C1) and the arteries (Fig. 2D1); the fluorescently-stained tissues of nerves were fibrous connective tissues as well, which were epineurium, perineurium and endoneurium respectively (Fig. 4A1, A2 and B). In contrast, the samples obtained from the skin and blood vessels at different sites of 6-7 cm away from the fluorescent pathways showed no sign of fluorescent staining.

The same frozen cross-sections of the tissue samples containing cutaneous and perivascular pathways were examined using the HE and the VG+VIH staining methods, confirming that these fluorescent pathways were fibrous connective tissues comprising a combination of abundant collagenous fibres and a few elastic fibres (Figs. 2A2, 2B2, 2C2, 2D2 and 4B). Each sample of the perivascular pathways and the cutaneous pathways was barely stained of D2-40.

3.4. Two-photon Laser Scanning Microscopy of the fluorescent pathways

TPLSM revealed the fluorescent cutaneous and the perivascular pathways in samples obtained from the dermis, hypodermis (Fig. 2A3), and the general loose connective tissues surrounding the veins (Fig. 2C3) and arteries (Fig. 2D3), comprising abundant fluorescently stained fibres (fibril bundles) running longitudinally along the long axis of the fluorescent pathways and having focal contacts with each other. In contrast, no fluorescent signals were observed in the dermis, hypodermis (Fig. 3A1, B1) and perivascular connective tissues (including the tunica adventitia) surrounding the veins (Fig. 3C1) and arteries (Fig. 3D1) in control subjects using TPLSM.

3.5. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) of the fluorescent pathways

The selected tissue samples prepared for SEM and TEM were the same as those obtained for the analysis of the frozen sections. The intrinsic solid structures within each fluorescent pathway observed using SEM were the longitudinal fibril bundles running along the long axis of the pathway (Fig. 2A4). By TEM, the fibrils exhibited period structures [7] and the fibril bundles were criss-crossed at micrometre scale (Fig. 3E1-2).

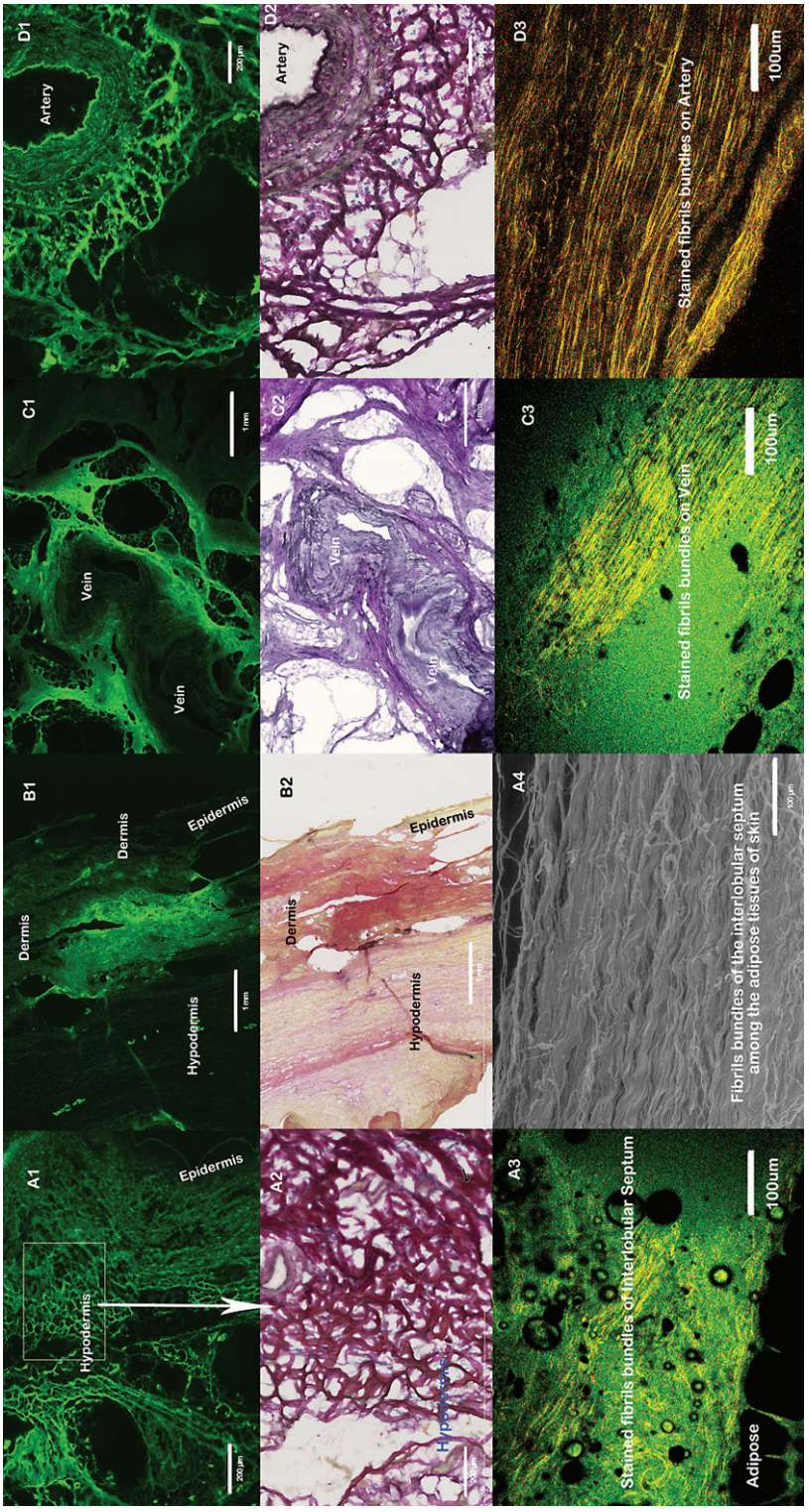

Fig. 2. Illustrations of the intrinsic structures of the cutaneous and perivascular pathways. Observed from the frozen cross-section view of fluorescence stereomicroscopy, the cutaneous path was mainly located in dermis and hypodermis (**A1**) and occasionally in dermis (**B1**) during the centripetal transportation. Accordingly, the perivascular paths were the stained tissues surrounding the venous vessel (**C1**) and arterial vessel (**D1**). The same frozen-sections were studied by the staining of VG+VIH and clearly showed that the cutaneous and perivascular paths were composed of the collagenous (pink-red) and elastic (black) fibers (**A2**, **B2**, **C2** and **D2**). The tissue samples of the frozen-sections were studied by TPLSM and showed that the cutaneous path (**A3**) and perivascular paths (**C3** and **D3**) were composed of the stained fibril bundles running longitudinally and having focal contacts with each other. By SEM, the fibril bundles of either the cutaneous paths or the perivascular paths were distributed along the long-axis of the paths and visualized similarly with the displayed longitudinal fibril bundles of interlobular septum among adipose tissues in skin (**A4**).

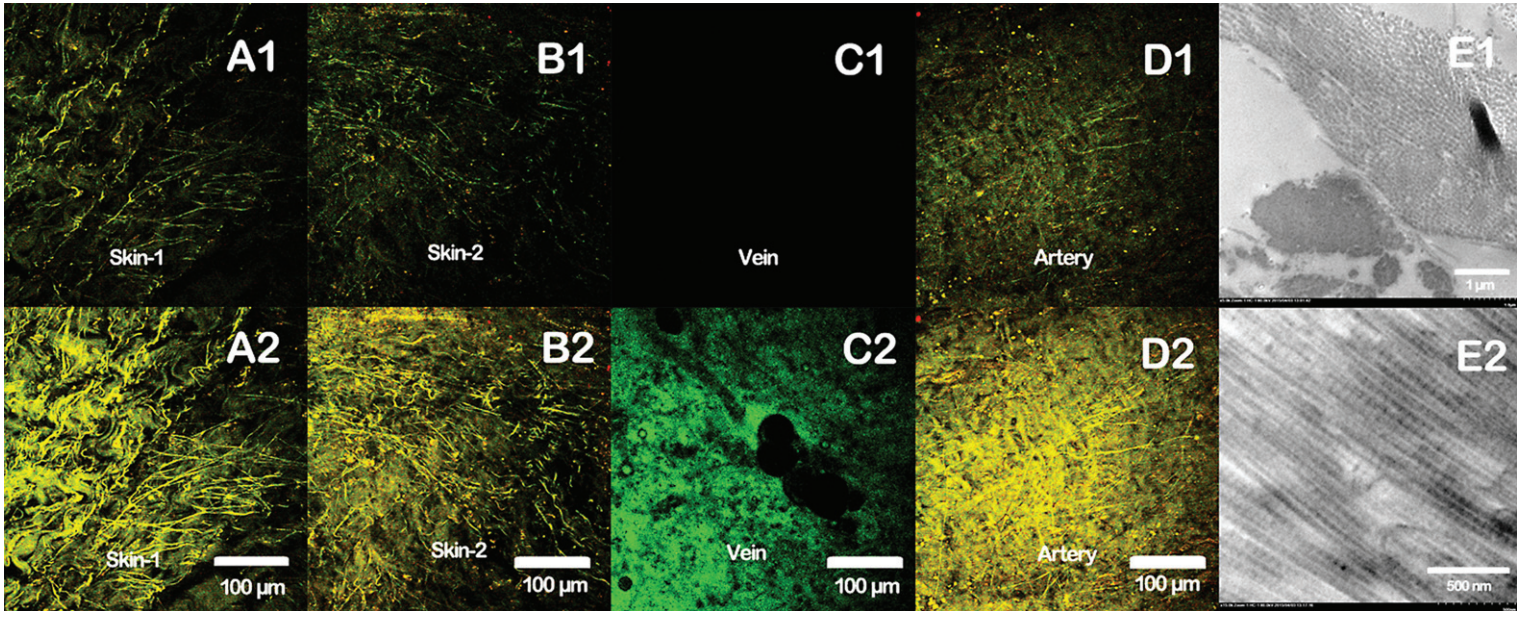

Fig. 3. Illustrations of the tissue **autofluorescence by TPLSM**. When imaging conditions were the same as Fig. **2A3**, **C3** and **D3**, fluorescent single of hypodermis (**A1** and **B1**), perivenous tissues (**C1**), and periarterial tissues (**D1**) were significantly weaker. To resolve the structure more evident, images **A2**-**D2** were taken with the same region corresponding to A1-D1 respectively but with higher excitation intensity (1.6 times). With this imaging condition, the intrinsic networks of the visualized fibril bundles within either hypodermis (**A2** and **B2**) or periarterial tissues (**D2**) were criss-crossed (the fibril bundles in perivenous tissues were invisible). All samples were taken from the skin and blood vessels away from the stained fluorescent paths (6–8 cm). **E1** showed the criss-crossed fibril bundles at micrometre scale by TEM. **E2** showed the periodic stripes in each fibril.

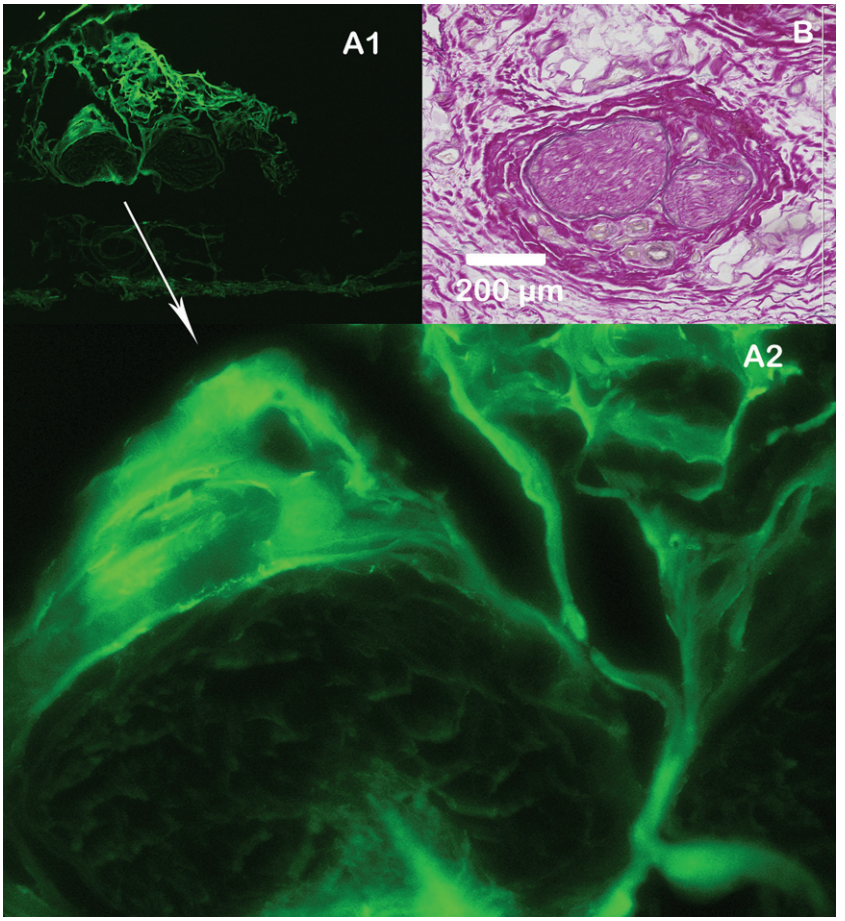

Fig. 4. Illustrations of two segments of nerves near the broken end in the patient who received the injections after the amputation. Observed by frozen fluorescence microscopy, two segments of nerve were stained by fluorescein from ankle (**A1**). Either the epineurium or perineurium or endoneurium was stained by fluorescein (**A2**). **B** showed the same nerve by VG+VIH staining.

4. Discussion

Clinically, edema manifests as dermal swelling; the increased secretion of fluid into the interstitium or the impaired removal of fluid can cause edema. Anatomically, the postcapillary venules and lymphatic vessels are essential for the removal of interstitial fluid and prevention of tissue edema. The accumulated fluid in skin would return into blood or lymphatic circulation by the Starling forces. In the first *in vivo* patient before the amputation, the injected NaF in ankle dermis should be drained physiologically into the blood and lymphatic vessels by the heart. However, the visualized fluorescent pathways originating from the injection point of ankle edema were neither blood nor lymphatic vessels, but an extravascular pathway of fibrous connective tissues. From the ankle to the broken end of the leg, the anatomical positions of the fluorescent pathways can be mainly divided into two types: the cutaneous pathways and the perivascular pathways (Fig. 1). In the long-distance course of centripetal transport, the cutaneous pathways consisted of the dermis (Fig. 2B1), the interlobular septum among the adipose tissues within the hypodermis (Fig. 2A1), while the perivascular pathways comprised

general loose connective tissues surrounding the veins (Fig. 2C1) and arteries (Fig. 2D1). By means of either fluorescence stereomicroscopy or the D2-40 staining that is supposed to highlight lymphatic vessels, none or bare lymphatic vessels were found in the sample of either the perivascular pathways or the cutaneous pathways. Limited by the anatomical methods, which one of the extravascular pathways or the vascular vessels was dominated in the drainage of the overloaded interstitial fluid in skin edema needed further studies.

Most interestingly, the revealed perivascular pathways surrounding the blood vessels, particularly the arteries, may suggest a perivascular fluid flow along the entire vascular trees in animals and have potential physiological or pathophysiological functions in vascular biology, such as the occurrence and development of atherosclerosis or even the thrombosis incidence (Fig. 2C1-3, D1-3).

Displayed by TPLSM, it was clearly visualized that both the cutaneous pathways and perivascular pathways were the fluorescently stained fibril bundles running longitudinally and having focal contacts with each other (Fig. 2A3, C3 and D3). The fluorescently stained extravascular pathways were actually the results of the fluorescent fluid flow to have stained the intrinsic fibril bundles of fibrous matrix.

Generally, interstitial fluid in normal conditions or extra fluid in dermis edema cannot flow freely through fibrous connective tissues according to common physiological knowledge. The interstitial fluid is thought to be entrapped by the twisted and thin coiled molecules of proteoglycans to form tissue gel and can flow freely only along the surfaces of collagen fibers or cells for a short distance before back to the circulations [6]. Within fibrous connective tissues, either the fibres and proteoglycan filaments or the hyaluronic acid and proteins are typically fixed relative to fluid flow [10]. This raised a question that whether there is a gap for fluid transport through the gel-like fibrous connective tissues.

In a view of porous medium, an interfacial zone between hydrophilic/hydrophobic solid fibril bundles and the liquid "tissue gel" surrounding them can be formed physically. In our recently published work, the intrinsic structures of loose connective tissues were fabricated by the poly (ε-caprolactone) (PCL) fibers embedded in polyacrylamide (PAM) gel. An interfacial (solid-liquid) zone between each PCL fiber and the surrounding liquid "hydrogel" was clearly revealed, contributing to a path of "fast molecular transport" [4]. By the VG+VIH staining, it was revealed that the fibril bundles comprised abundant hydrophilic collagenous fibril bundles and a few hydrophobic elastic fibers (Figs. 2A2, 2B2, 2C2, 2D2 and 4B), indicating that the solid fibril bundles contained the hydrophilicity and hydrophobicity to some extent. Therefore, a gap/space within fibrous connective tissues, namely a solid-liquid interfacial zone, can be formed physically between individual fibril bundle and the surrounding gel-like ground substance and responsible for the staining process of fluorescein through fibrous connective tissues. The detailed features and kinetic mechanism of fluid transport through the long-distance fibrous pathways in humans required further studies.

In the other three amputated lower legs, both the blood and lymphatic circulation were terminated for more than 2 hr at least. The fluorescein in the dorsum only diffused around the injection site due to the local concentration gradient of the injected fluorescein physically, and there were no signs of the long-distance longitudinal fluorescent pathways without external interferences.

In physiology, the movements of blood, lymph and the cerebrospinal fluid through the vessels and the perivascular spaces can be driven by the muscle constrictions and arterial pulsations, as well as artificial compression of enhanced external counterpulsation (EECP) [2, 3, 5, 15, 18]. Here, we hypothesized that the mechanical movements of the muscle tissues can generate some kinetic forces that were responsible for the staining process of fluorescein through the unique fibrous pathways. By repeated cyclical compression, the mechanical effects on the broken end of the lower legs succeeded to "pull", but not "push", fluid from the cutaneous and perivascular pathways for a long-distance distance of more than 10 cm, in accordance with those observed in the *in vivo* analyses. However, the limbs of the *in vivo* volunteer were paralysed due to the epidural anesthesia and did not move mechanically

after the subcutaneous injection into the point. Considering the blood vessels are connected with the heart, whether the mechanical movements of the heart were one of the *in vivo* causes of "pulling" fluid through the extravascular pathways, like our previous findings of the pericardial fluid formation by the PLCTs in rabbits, needs further studies.

Observed by the combined staining with VG+VIH or TEM, the orientations of the micron-sized fibril bundles (comprising collagenous and elastic fibres) within the fluorescent fibrous pathways were nearly isotropic at three dimensions (Figs. 2A2, 3E1-2). However, only the longitudinally distributed fibril bundles along the long axis of the cutaneous or perivascular pathways could be stained by fluorescein (Figs. 1A, 2A3, 2C3 and 2D3). Presumably, it was necessary for the unique fluid transportation that the interfacial zones along the intrinsic fibril bundles of fibrous matrix should be fully interconnected and longitudinally running throughout the entire long-distance fibrous pathways.

In anatomy, the perivascular pathways in humans would possibly converge into the grooves of the heart like the rabbits in that the PLCTs pathways contributed to the formation of pericardial fluid. As to the cutaneous pathways, their destinations need to be identified. Intriguingly, the presented anatomical method might provide the clues to clarify the panorama of anatomical distributions of the long-distance fibrous pathways by artificial heart beatings and/or respiratory mechanical ventilation in human dead body, including the skin, the extremities, the muscles, various visceral organs and even the peripheral and central nervous system.

In the vicinity of the perivascular pathways of lower legs, the epineurium, perineurium and endoneurium of two nerves were stained by the fluorescein from ankle as well (Fig. 4), which may strongly suggest fluid flow through the fibrous matrix of nerves and inspire a novel area of neuroscience, for instance, contributing to the explorations on the causes of neurodegenerative diseases and etc.

In summary, the anatomical findings obtained from human amputee subjects testified that an oriented fibrous connective tissue constitutes a long-distance non-vascular fluid transport pathway. Through the interfacial zones of these fibrous pathways, named "a biotic interfacial transport pathway (BIFT pathway)", it is likely in animals that fluids within fibrous matrix in any tissues or organs are not fixed within "tissue gel" but driven towards designed destinations via various as yet unidentified physical mechanisms. The rheology of fluid in the interstitial spaces of skin and perivascular spaces or even other fibrous tissues should be reevaluated in terms of considering fluid transport through the interfacial zones within the gel-like fibrous connective tissues. In addition to the drainage of interstitial fluid or the compensation for ankle dermal edema, the challenges to comprehensively understand the biotic interfacial kinetics, the visualization and characterizations, the physiological and pathophysiological functions of fluid transportation through the BIFT pathways would attract further research attentions in life science.

Acknowledgments

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Disclosure

The authors declare no competing financial interests and have no conflicts to disclose.

Contributions

Conceived and designed the experiments: HyL, DH. Performed the experiments: HyL, QcY, LrD. Analyzed the data: HyL FW. Contributed reagents/materials/analysis tools: KyL, LyZ, JFY, FW, DgL. DC, MjS, JxP. Wrote the paper: HyL, DH, approval of the article: HyL.

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