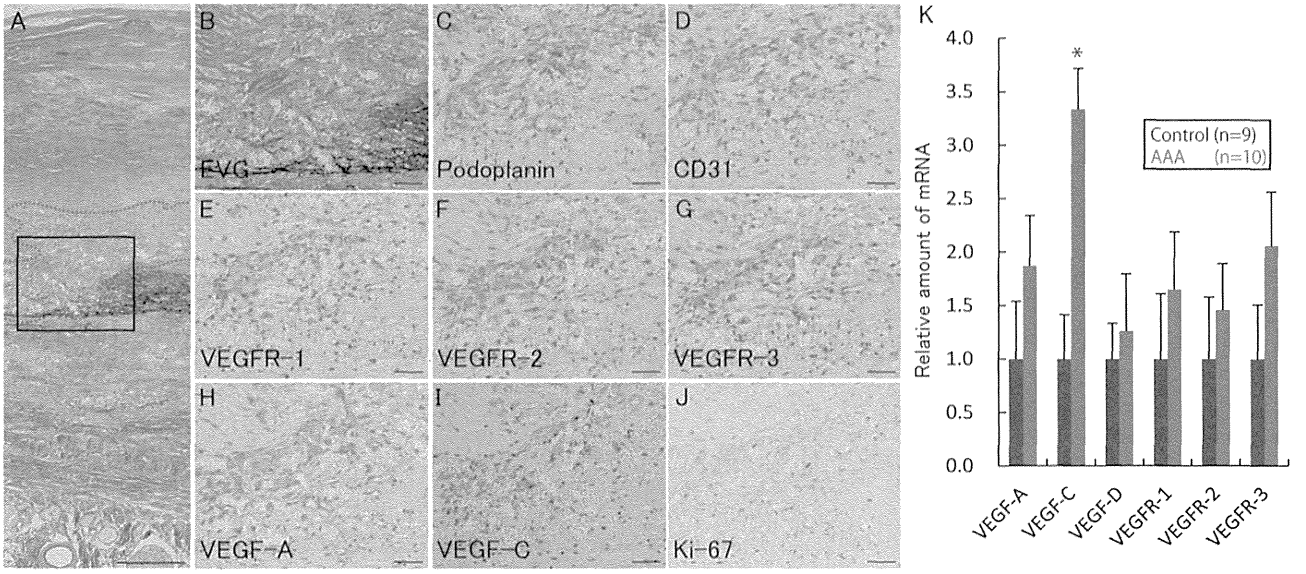

Figure 2. Lymphangiogenesis and angiogenesis in abdominal aortic aneurysm walls. A, Elastica van Gieson staining of the abdominal aortic aneurysm (AAA) wall. Microvessels in the intima/media are encircled by a red dotted-line. **B**, Higher magnification of the area outlined with a solid line in **A**. Immunohistochemistry for podoplanin (**C**), CD31 (**D**), VEGFR-1 (**E**), VEGFR-2 (**F**), VEGFR-3 (**G**), VEGF-A (**H**), VEGF-C (**I**), and Ki-67 (**J**) in the area outlined with a solid line in **A**. The microvessels encircled by the red dotted-line consisted of podoplanin-positive cells (**C**) and CD31-positive cells (**D**). These microvessels were positive for VEGFR-1, VEGFR-2, and VEGFR-3 (**E–G**). Expression of VEGF-A and C was increased within and around these microvessels (**H, I**). The nuclei of these microvessels were positive for Ki-67 (**H**). **K**, Comparison of mRNA expression of VEGF-A, VEGF-C, VEGF-D, VEGFR-1, VEGFR-2, and VEGFR-3 between normal aorta and AAA. Data were obtained from 10 AAA patients and 9 autopsied cases (controls). Data were analyzed by comparative Ct method. All of the mRNAs except VEGF-D were upregulated in AAA tissues. Standard deviation is indicated by bar errors. * indicates $p<0.05$ vs control. Scale bars indicated 200 μm (**A**), 50 μm (**B–J**).
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within and around lymphatic microvessels in both intima and adventitia of AAA walls. However, LYVE-1-positive macrophages were only observed in intima, not in adventitia. Previous studies have reported that macrophages are related to lymphangiogenesis in pathological processes such as malignant tumors [23], inflammation [24–26], and transplantation [27]. Macrophages are thought to support lymphangiogenesis in two ways: by transdifferentiation into lymphatic endothelial cells [23,25–27], and by stimulating preexisting local lymphatic endothelial cells via release of lymphangiogenic factors [24,25].Although LYVE-1 is regarded to be specifically expressed by lymphatic endothelial cells [20], LYVE-1-positive macrophages have also been reported in

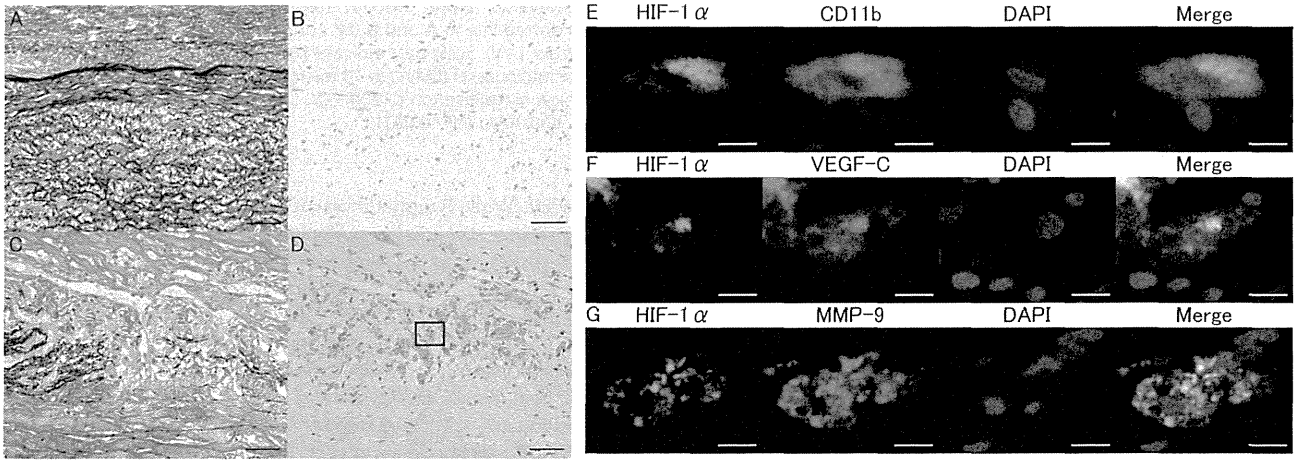

Figure 3. HIF-1 expression in normal aorta and AAA. Elastica van Gieson staining of the intima/media in normal aorta (**A**) and abdominal aortic aneurysm (AAA) wall (**C**). Immunohistochemistry for HIF-1α in normal aorta (**B**) and AAA (**D**). Nuclear and cytoplasmic expression of HIF-1α was observed in intima/media in AAA (**D**). **E**, Double immunofluorescence staining for HIF-1α (green), CD11b (red), DAPI (blue), and the merged image of the outlined in **D**. **F**, Double immunofluorescence staining for HIF-1α (green), VEGF-C(red), DAPI(blue), and the merged image of the outlined in **D**. **G**, Double immunofluorescence staining for HIF-1α(green), MMP-9 (red), DAPI (blue), and the merged image of the outlined in **D**. Expression of HIF-1α was increased in CD11b positive macrophages. VEGF-C and MMP-9 were expressed in the HIF-1α–positive macrophages. Scale bars indicated 50 μm (**A–D**) and 10 μm (**E–G**).
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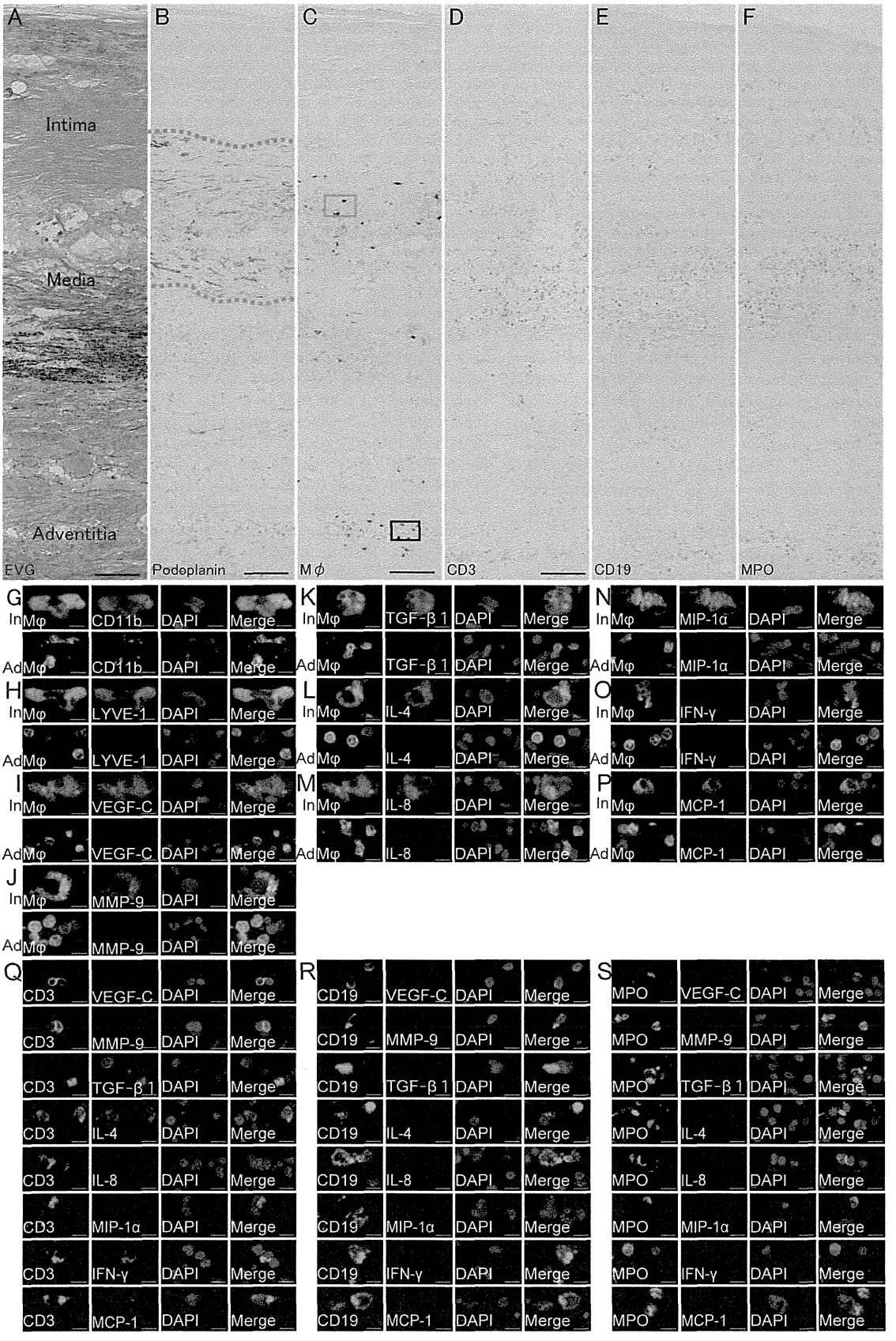
A
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Figure 4. Macrophage infiltration of AAA microvessels. A, Elastica van Gieson staining of abdominal aortic aneurysm (AAA). Immunohistochemistry for podoplanin (**B**) and macrophages (**C**), CD19 (**E**), and myeloperoxidase (MPO) of the AAA wall. **B**, Lymphatic microvessels in the intima/media of the AAA (red dotted line encircling lymphatic microvessels). **C**, Macrophages infiltration around/within lymphatic microvessels in the intima/media or in adventitia (red square: macrophages in intima/media, black square: macrophages in adventitia). **D**, CD3-positive T cells infiltration around/within lymphatic microvessels in the intima/media or in adventitia. **E**, CD19-positive B cells infiltration around/within lymphatic microvessels in the intima/media or in adventitia. **F**, MPO-positive neutrophils infiltration in the intima/media or in adventitia. **G–P**, Double immunofluorescence staining of macrophages infiltrating the intima/media (In) and adventitia (Ad). **G–P**, Macrophage: green, CD11b (**G**)/LYVE-1 (**H**)/VEGF-C (**I**)/MMP-9 (**J**)/TGF-β1 (**K**)/IL-4 (**L**)/IL-8 (**M**)/MIP-1α (**N**)/IFN-γ (**O**)/MCP-1 (**P**): red, DAPI: blue. LYVE-1, VEGF-C, MMP-9, TGF-β1, IL-4, IL-8, MIP-1α, and MCP-1 were expressed in the CD11b-positive macrophages in the intima/media, but not by macrophages in adventitia. **Q**, Double immunofluorescence staining of T-cells in intima/media and inflammatory cytokines. CD3: green, VEGF-C/MMP-9/TGF-β1/IL-4/IL-8/MIP-1α/IFN-γ/MCP-1: red, DAPI: blue. TGF-β1, IL-4, and IFN-γ were expressed in CD3-positive T lymphocytes in the intima/media. **R**, Double immunofluorescence staining of B lymphocytes in the intima/media and inflammatory cytokines. CD19: green, VEGF-C/MMP-9/TGF-β1/IL-4/IL-8/MIP-1α/IFN-γ/MCP-1: red, DAPI: blue. These inflammatory cytokines were not expressed in CD19-positive B lymphocytes. **S**, Double immunofluorescence staining of neutrophils in intima/media and inflammatory cytokines. MPO: green, VEGF-C/MMP-9/TGF-β1/IL-4/IL-8/MIP-1α/IFN-γ/MCP-1: red, DAPI: blue. These inflammatory cytokines were not expressed in MPO-positive neutrophils. Scale bars indicated 100 μm (**A–F**) and 10 μm (**G–S**).
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inflammatory tissue and malignant tumors [23,26,27]. Maruyama et al. recently reported that CD11b positive macrophages expressed LYVE-1 under inflammatory conditions in the cornea of mice. These macrophages aggregated and formed vessel-like structures, that expressed lymphatic endothelial cell markers, such as LYVE-1, Prox-1, and podoplanin [26]. CD11b and LYVE-1-positive macrophages may transdifferentiate into lymphatic endothelial cells. These macrophages could be derived from the circulation and transmigrate through the connective tissues, contributing to de novo lymphangiogenesis [25–27].

In the present study, overexpression of HIF-1α was observed in the intima/media of AAA, and large macrophages infiltrated hypoxic areas in the region. As we mentioned previously, the infrarenal aortic wall is susceptible to hypoxia. The presence of intraluminal thrombus (ILT) may prevent luminal perfusion of oxygen, allowing only the adventitial VV to deliver oxygen and nutrients to the aortic wall. Moreover, we recently reported that adventitial VV becomes stenotic due to the intimal hyperplasia, which causes malperfusion of the AAA wall and tissue hypoxia [5]. Notably, the tissue in the intima/media of AAA with thick ILT is farthest from both luminal perfusion and VV perfusion, so those layers are particularly prone to hypoxia. Vorp et al. also reported that thicker ILT was associated with localized tissue hypoxia, neovascularization, and inflammation in the AAA wall [28]. We therefore speculate that LYVE-1-positive macrophages may be induced by hypoxia in aortic intima/media and may transdifferentiate into lymphatic endothelial cells following lymphangiogenesis.

Macrophages can also promote lymphangiogenesis by producing lymphangiogenic factors, thereby stimulating the preexistent lymphatic endothelial cells in inflammatory tissue or in malignant tumors [23,24]. The development of lymphatic vessels is regulated either by lymphangiogenesis-specific or angiogenesis-nonspecific factors in malignant tumors [24,29]. Macrophage infiltration stimulates lymphatic endothelial cells by releasing VEGF-C. VEGF-C/VEGFR-3 signaling plays a critical role in the growth and survival of lymphatic endothelial cells in inflammation or in malignant tumors [25,29]. In the present study, lymphatic microvessels were observed in the intima/media of AAA walls, where mRNA expressions of both VEGF-C and VEGFR-3 were more prominent. Expression of VEGF-C was also increased in HIF-1α positive macrophages infiltrating the intima/media but not in adventitial macrophages. Furthermore, the expression of VEGFR-3 was increased in areas of neovascularization in the intima/media of AAA walls. Taken together, these results suggested that hypoxia-induced infiltration of macrophages in intima/media may release VEGF-C, stimulating the lymphatic endothelial cells and thus contributing to lymphangiogenesis.

In this study, VEGF-A positive cells were also observed within and around the microvessels in both intima and media of AAA walls. The mRNA expression of VEGF-A and VEGFR-1 was more prominent than that in normal aorta. Kaneko et al. also reported VEGF-A overexpression in the macrophages infiltrating AAA walls [30]. Because VEGF-A is not only a potent angiogenic factor but also promotes lymphangiogenesis [24,29], the VEGF-A/VEGFR-1 signaling pathway may also play an important role in both angiogenesis and lymphangiogenesis in the AAA wall.

Infiltration of inflammatory cells such as macrophages, T and B lymphocytes, and neutrophils, have been identified in AAA specimens [31]. In atherosclerotic lesions, monocyte-derived macrophages and T lymphocytes were observed most often [32,33]. During each phase of atherogenesis, the inflammatory response is mediated by monocyte-derived macrophages and specific subtypes of T lymphocytes [34]. With regard to macrophages, Stoger et al. reported that, atherosclerotic lesions contain foamy large macrophages expressing M1 markers in the intimal plaque, while adventitial small macrophages express M2 signature karkers [35]. Similarly, in AAA specimens, both macrophages and several subtypes of T lymphocytes also have been identified [31]. In this study, the infiltration of large macrophages and T lymphocytes was confirmed in the intima/media, while small macrophages and T lymphocytes were observed in the adventitia of AAA. Only large macrophages in the intima/media of AAA expressed VEGF-C and MMP-9. Large macrophages may be associated with hypoxia in the intima/media, contributing to lymphangiogenesis and the progression of AAA. On the other hand, T lymphocytes and adventitial small macrophages did not express either VEGF-C or MMP-9, suggesting that T lymphocytes might not play a major role in involved in lymphangiogenesis in AAA walls. Infiltration of T and B lymphocytes and neutrophils was markedly observed around lymphatic microvessels, suggesting that formation of lymphatic microvessels may introduce these inflammatory cells into the intima/media. Macrophages in the intima/media expressed not only VEGF-C and MMP-9 but also TGF-β1, IL-4, IL-8, and MIP-1α. These cytokines are known to cause further recruitment of macrophages and neutrophils, potentiating inflammation [31,36]. On the other hand, T lymphocytes expressed TGF-β1, IL-4, and IFN-γ but not VEGF-C and MMP-9, which suggested that T lymphocytes might accelerate macrophage recruitment in the region, but not directly contribute to the lymphangiogenesis [37,38]. Inflammatory cytokines such as TGF-β1, IL-4, IL-8, and IFN-γ were reported to promote lymphangiogenesis in various diseases [39] [40] [41] [42]. Therefore, cytokines such as TGF-β1, IL-4, IL-8, and IFN-γ may also contribute to lymphangiogenesis in AAA. With regard to B lymphocytes in the intima/media, neither VEGF-C, MMP-9, TGF-β1, IL-4, IL-8, MIP-1α, IFN-γ,

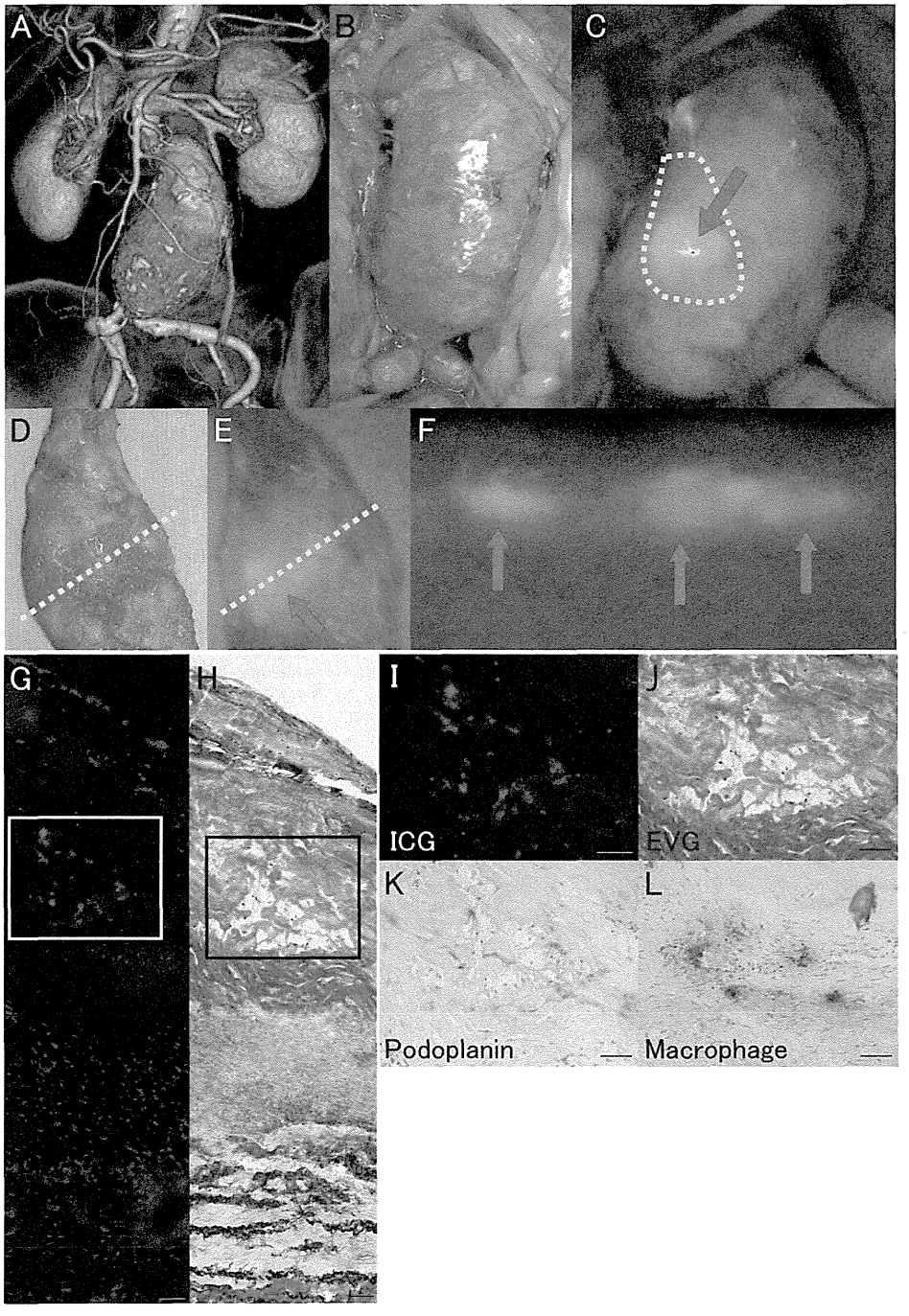

Figure 5. Fluorescence lymphography and microscopy of abdominal aortic aneurysm. A, Three dimensional CT image of an infrarenal abdominal aortic aneurysm (AAA). **B**, Intraoperative photography of an infrarenal AAA. **C**, Intraoperative near-infrared fluorescence lymphography of indocyanine green (ICG) (red arrow: ICG fluorescence, yellow dotted-line: sample resection line). **D**, Macroscopic findings of the resected sample observed from the luminal side (yellow dotted line: cross-section line of the harvested sample). **E**, Near-infrared fluorescence lymphography of ICG in the resected aneurysmal wall observed from the luminal side (red arrow: ICG fluorescence, yellow dotted line: cross-section line of the harvested sample). **F**, Near-infrared fluorescence lymphography of ICG in the cross-sectioned AAA wall (red arrow: ICG fluorescence, up: luminal side, bottom: adventitial side). **G**, Fluorescence microscopy of ICG in the AAA wall (ICG: red, DAPI: blue). **H**, Serial frozen section with Elastica van Gieson staining. **I**, Higher magnification of the outlined area in **G**. **J**, Higher magnification of the outlined area in **H**. **K**, Immunohistochemistry for podoplanin in the outlined area in **G** and **H**. **L**, Immunohistochemistry to label macrophages in the outlined area in **G** and **H**. Scale bars indicate 50 μm (**G–L**).
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nor MCP-1 were expressed, which suggested that B cells may not play a major role in lymphangiogenesis in AAA walls. Neutrophils did not express either VEGF-C and MMP-9, suggesting that neutrophils may contribute to macrophage recruitment rather than lymphangiogensis itself.

We investigated lymph stasis in AAA patients using ICG fluorescence lymphography. ICG fluorescence lymphography is an emerging technique to enable intraoperative observation of lymphatic flow [43,44]. In the present study, ICG fluorescence dye, which was subcutaneously injected bilaterally into the dorsum of the foot, stagnated in the AAA wall. Intraoperative ICG lymphography using a near-infrared camera visualized marked fluorescence signals in the AAA wall after exposure of the retroperitoneal space. Freshly harvested samples of the cross-sectioned AAA walls also demonstrated fluorescence signals in the intima/media, which was later confirmed using ICG fluorescence microscopy. These results suggest that lymph stasis occurred in the intima/media of AAA wall. This suggested that neovascularized lymph vessels were a less efficient lymph-transport pathway, causing lymph stasis in the intima/media of AAA wall. In the harvested samples, the lymphedematous regions in the AAA wall also demonstrated marked infiltration of macrophages. Previous studies have associated chronic lymph stasis with tissue inflammation [45]. Recently, Zampell et al. reported that lymph stasis causes infiltration of mononuclear cells and tissue fibrosis in a lymphatic fluid stasis model using rat tails [46,47]. Therefore, AAA wall lymphatic dysfunction causes lymph fluid stasis, which may contribute to the medial inflammation in the AAA wall [47]. Hypoxia-induced lymphangiogenesis could also be enhanced by lymph stasis [48]. However, the role of lymphangiogenesis in the AAA wall is unknown. Angiogenesis is thought to contribute to destructive processes within the AAA wall and plays a key role in aortic aneurysm development and rupture [7]. Therefore, various anti-angiogenic agents have been studied in animal models for potential treatment of AAA [30,49,50]. Maruyama et al. reported that down-regulation of VEGFR-3 and subsequent inhibition of lymphangiogenesis delayed diabetic wound healing in a mouse model [51]. Moreover, Zhou et al. reported that administration of VEGF-C and increased lymphangiogenesis improved lymph drainage and inhibited inflammation in a rat model of chronic arthritis [52]. Thus, inflammation has been widely accepted to stimulates lymphangiogenesis as a compensatory mechanism to enhance the clearance of inflammatory products. Therefore, lymphanagiogenesis in the AAA wall may be a rational response against loss of adventitial LVV and lymph stasis. However, as we demonstrated in the present study using ICG fluorescence lymphography, the newly formed lymphatic vessels may not fully function to drain lymph. Then, the insufficient lymph drainage may provoke further inflammation.

In conclusion, macrophage infiltration may be associated with lymphangiogenesis and angiogenesis in the intima/media. Lymph-drainage appeared to be insufficient in the AAA wall, which may become a new therapeutic target for non-surgical treatment of AAA by improving lymph flow.

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Author Contributions

Conceived and designed the experiments: NU M. Sano TS SH MO. Performed the experiments: M. Sano TS SH JS NZ HT. Analyzed the data: M. Setou KS HK NU. Contributed reagents/materials/analysis tools: SH JS SB KI NY. Wrote the paper: M. Sano NU. Patients recruit and registration: KI NY.

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Lymphatics in nanophysiology ☆

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Cancer

ABSTRACT

Lymphatic vessels are essential for fluid transport and tissue homeostasis. Recent discoveries identified several genes, including Prox1 and VEGF-C, which are required for the lymphatic vessel development in physiological conditions as well as under pathological conditions such as chronic inflammation and tumor progression. Lymphatic vessels show morphological structures that are distinct between the initial lymphatic vessels and collectors, reflecting their respective functions of fluid absorption and transport. These differential structures are crucial for the physiological function of lymphatic vasculature. VEGF-A-mediated chronic inflammation impairs the fundamental structure of the initial lymphatic vessels, leading to delayed transport of nano-scaled fluorescence tracers. This article discusses recent findings that have clarified the biological function of lymphatic vessels in physiological and pathological settings. Assessments of the lymphatic function at nano-scale levels address the major contribution of lymphatic vessels to the kinetics of drug delivery and excretion.

Contents

1. Introduction

Lymphatic vessels contribute to the function of the lymphatic system in the mammalian body. The distinct structure at the cellular level allows lymphatic vessels to absorb the interstitial fluid (lymph) in peripheral organs and to drain lymph to the blood circulation. Lymphatic vessels play an essential role in promoting active immune surveillance in conjunction with lymph nodes [1]. New lymphatic vessel growth (called lymphangiogenesis) is induced in several pathological conditions such as chronic inflammation or tumor progression. Emerging evidence from experimental animal models shows that lymphatic transport is impaired in chronic inflammation [2]. Tumor-associated lymphangiogenesis promotes enhanced metastasis to draining lymph nodes and beyond in experimental tumor models [3–7]. Importantly, tumor lymphangiogenesis is also associated with reduced patient survival in several types of human cancer [8–11].

Recent studies indicated that pathological lymphatic vessel growth is mediated by several factors such as pleiotropic growth factors, pro-inflammatory cytokines and inflammatory cells such as macrophages [12,13], whereas physiological lymphangiogenesis requires vascular endothelial growth factor (VEGF)-C [14]. These molecules and inflammatory cells likely contribute to the biological alteration of lymphatic vessels under pathological conditions. However, it remains unclear if and how lymphatic vessels are structurally or functionally altered

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during pathological processes such as chronic inflammation and tumor progression.

Effective drug delivery is mediated by blood flow to specific sites where pathological alterations develop. Furthermore, regulation of vascular permeability is required for efficient drug delivery to the microenvironment. Recent advances based on nano-scaled carriers make it now possible to assess the biological function of blood vessels by quantitative measurement of plasma leakage. In contrast, it remains unclear whether lymphatic vessels contribute to the delivery of drugs and their excretion in pathological conditions such as chronic inflammation and tumor progression. This article will review the recent progress of research with regard to the fundamental role of lymphatic vessels in drug delivery and will further focus on the biological importance of lymphatic vessels in disease progression.

2. Blood and lymphatic vessels in the skin

Blood flow, the major circulation in the mammalian body, delivers oxygen, nutrients and cells to the peripheral organs. In the skin, blood vessels underlie the epidermis that represents a shield for the whole body (Fig. 1A). Epidermal keratinocytes and melanocytes – the major components of the epidermis – are supplied by blood capillaries that physiologically leak plasma into the interstitial space. Conversely, lymphatic vessels absorb interstitial tissue fluid and macromolecules that arise from the epidermal cells in physiological conditions (Fig. 1A) and further play a key role in promoting immune cell responses in pathological conditions such as inflammation and tumor progression (Fig. 1B).

3. Key regulators in physiological lymphangiogenesis and lymphatic disorders

Physiological lymphatic vessel development has two quintessential regulators. Prox1, a homeodomain transcription factor, induces the reprogramming of cell fate from venous endothelial cells to lymphatic progenitors [15]. VEGF-C – a key growth factor – promotes physiological lymphangiogenesis by the activation of VEGFR-3, a receptor tyrosine kinase expressed in lymphatic endothelial cells [16,17]. The importance of these factors is reflected by the impairment of lymphatic vessel function and the formation of lymphedema or chylous ascites after genetic inactivation. Targeted deletion of the genes encoding *Prox1* or *Vegfc* in mice resulted in lethal phenotypes of null mice during embryonic development, due to the lack of lymph vessels and the formation of severe lymphedema [14,18]. Furthermore, a heterozygous *Vegfc* mutation leads to chylous ascites as well as lymphedema in *Chy-3* mice, due to hypoplasia of lymphatic vessels [19]. Importantly, a missense mutation in the VEGFR-3 tyrosine kinase domains is responsible for Milroy's disease, a familial lymphedema that develops in patients after birth. Moreover, functional inactivation of a single allele of *Prox1* led to adult-onset obesity as well as lymphedema due to abnormal leakage of lymph from lymphatic vessels [20]. These findings indicate that Prox1 and VEGF-C play essential roles in promoting lymphatic vessel development in physiological settings.

4. Lymphatic vessel patterns in physiological conditions and lymphatic disorders

Lymphatic vessels in the skin begin with lymphatic capillaries in the papillary dermis (Fig. 2A). The initial lymphatic vessels are blunt-ended and are composed of partly-overlapping lymphatic endothelial cells (Fig. 2B). The characteristic feature of the initial lymphatic vessels is represented by 'button-like junctions' that form microvalves between the endothelial cells [21]. Importantly, these lymphatic endothelial cells show discontinuous cell adhesion and basement membranes, leading to efficient absorption of the interstitial tissue fluid into the lymphatic vessels. The endothelial cells are attached by anchoring filaments, which contribute to solid cell adhesion to the extracellular matrix in the skin (Fig. 2B).

Lymphatic vessels are characterized by their expression of specific markers, which are distinct from blood vessel-specific molecules. Lymphatic vessel endothelial hyaluronan receptor (LYVE)-1, a type I transmembrane protein, is exclusively expressed by lymphatic endothelial cells in the initial lymphatic vessels [22]. Therefore, LYVE-1 is the most useful marker for the lymphatic capillaries in the skin (Fig. 2C). Podoplanin, a cell surface glycoprotein, is expressed in lymphatic capillaries as well as in collecting lymphatic vessels (Fig. 2C–E). The lymphatic collectors are characterized by the tight cell adhesion between lymphatic endothelial cells, the so-called 'zipper-like junctions' [21]. The collecting lymphatic vessels are further characterized by the presence of intraluminal valves and sparse coverage with pericytes. Pericytes express α-smooth muscle actin, which mediates the physiological contractions of the collecting lymphatic vessels (Fig. 2E). Thus, the pericytes and valves contribute to an efficient transport of lymph from proximal sites.

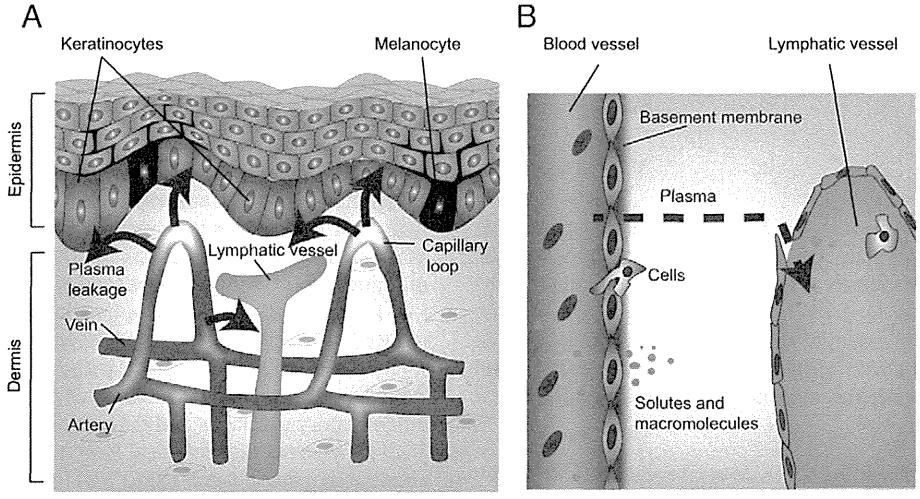

Fig. 1. Structure and function of blood and lymphatic vessels in the skin. (A) Blood vessels underlie the interfollicular epidermis. Blood capillaries leak plasma under physiological conditions, supplying oxygen and nutrients to the overlying epidermal keratinocytes. Cutaneous veins return blood *via* the subcutaneous tissue to the systemic circulation, whereas the lymphatic vessels begin with a blunt end in the skin. (B) Physiological interaction of cutaneous blood and lymphatic vessels. Interstitial tissue fluid contains plasma as well as solutes and macromolecules, which are secreted by several types of cells in the skin. Initial lymphatic vessels take up the fluid and macromolecules in addition to immune cells, maintaining the tissue homeostasis and immune-surveillance.

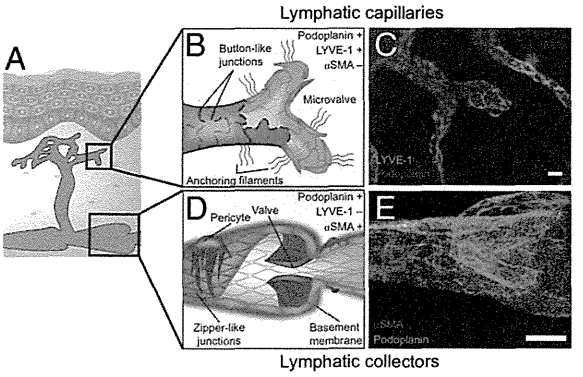

Fig. 2. Differential structure and function of initial and collecting lymphatic vessels. (A) Representative distribution of initial and collecting lymphatic vessels in the skin. (B) Initial lymphatic vessels are attached by anchoring filaments to the dermis and have microvalves, which allow efficient absorption of fluid and macromolecules from the interstitial space in the dermis. The cell-to-cell adhesion is characterized by discontinuous 'button-like junctions' that enable lymphatic endothelial cells to efficiently absorb and retain the interstitial tissue fluid. (C) Double immunofluorescence stains for LYVE-1 (green) and podoplanin (red) show that the initial lymphatic vessels are positive for both lymphatic vessel-specific markers. (D) Representative scheme of the collecting lymphatic vessels. Lymphatic collectors develop valves that allow uni-directional lymphatic flow. Collecting lymphatic vessels are covered by basement membranes and pericytes that mediate efficient lymphatic flow in the skin. (E) Double immunofluorescence stains for podoplanin (green) and α-smooth muscle actin (red) show that pericytes play a key role in promoting the efficient lymphatic flow by the collecting lymphatic vessels. Scale bars, 20 μm (C, E).

Valve formation and pericyte recruitment are highly organized processes during lymphatic vessel development in mammalian embryos. Foxc2, a forkhead family transcription factor, plays a key role in promoting the valve formation and pericyte recruitment by collecting lymphatic vessels during physiological lymphangiogenesis. Functional inactivation of *Foxc2* in mice led to the lack of valves in the collecting lymphatic vessels and abnormal accumulation of pericytes around lymphatic capillaries [23]. The abnormal lymphatic vessels showed a marked impairment of fluid transport, leading to the formation of lymphedema during embryonic development [23]. In humans, *FOXC2* mutations are responsible for the lymphedema distichiasis syndrome, which shows a decrease of fluid uptake by initial lymphatic vessels in the affected extremities, in addition to an extra row of eyelashes, with an autosomal dominant pattern of inheritance [24,25]. The affected skin of patients with lymphedema distichiasis syndrome shows abnormal pericyte coverage of the initial lymphatic vessels [23]. Therefore, the regulation of pericyte recruitment plays a fundamental role in promoting fluid uptake and drainage by the lymphatic vessels, contributing to tissue homeostasis in the skin and other affected tissues.

5. Impaired fluid transport in VEGF-A-mediated chronic inflammation

Pathological lymphatic vessel growth requires several factors such as pleiotropic growth factors, pro-inflammatory cell types and growth factors in addition to VEGF-C that promotes physiological lymphangiogenesis [26]. Targeted overexpression of VEGF-A in mouse skin promotes the persistent swelling of affected tissues and the enlargement of lymphatic vessels, in addition to the angiogenesis, in the chronic inflammatory tissue response that resembles psoriasis [2,27,28]. Scanning electron microscopy analysis showed that the lymphatic endothelial cells of initial lymphatic vessels form 'button-like junctions' in wild-type mice (Fig. 3A). In contrast, targeted overexpression of VEGF-A in mouse skin induced a chronic inflammatory tissue response, led to prolonged tissue swelling and reduced cell adhesion in the initial lymphatic vessels (Fig. 3B). Small to large-sized fenestrations were found in the cutaneous lymphatic capillaries in VEGF-A transgenic mice. Lymphatic capillaries have perforations in their basement membranes due to discontinuous expression of basement membrane components such as type IV collagen [29]. The LYVE-1-positive endothelial microvalves in lymphatic capillaries further allow immune cells as well as nano-sized small particles to enter the lymphatic lumen (Fig. 3C). Of particular importance, the lymphatic vessel drainage function is impaired during chronic skin inflammation in VEGF-A transgenic mice [2]. Cutaneous delayed-type hypersensitivity reactions were induced and monitored for two weeks in the ears of keratin 14 promoter-driven VEGF-A transgenic mice. These mice were subjected to intradermal injection of indocyanine green (ICG)-containing liposomes into the ear skin and were monitored for their lymphatic drainage to the superficial cervical lymph nodes. In uninflamed mice, the nano-scaled ICG particles were absorbed into the initial lymphatic vessels because microvalves were functional in the 'button-like junctions' (Fig. 3C). In contrast, a significant delay of the lymphatic transport was found in the skin of inflamed VEGF-A transgenic mice. Due to the persistent inflammation, loss of cell adhesion induced the impairment of the microvalves and dysfunction of fluid absorption and retention within the initial lymphatic vessels (Fig. 3D). While nano-scaled ICG liposomes rapidly flowed through the draining lymph nodes in uniflamed mice (Fig. 3E), flow of the tracer was severely delayed in VEGF-A transgenic mice (Fig. 3F). Thus, targeted overexpression of VEGF-A in mouse skin facilitates tissue inflammation, leading to the structural alteration and functional impairment of the initial lymphatic vessels. Importantly, the persistent inflammation may also impair normal transport of specific antigens from skin to draining lymph nodes, leading to an incomplete immune activation. Considering the potential impact on drug delivery, the reduced drainage of a drug might increase the time of exposure to a specific site of chronic inflammation.

6. Role of tumor lymphangiogenesis and its biological impact on tumor metastasis

Sentinel lymph node metastasis often indicates the initial spread of malignant tumors from the primary site. Furthermore, lymph node metastases in distant sites represent disease progression in certain types of cancer. The potential impact of lymph node metastasis on patient survival is well documented in malignant neoplasms such as cutaneous malignant melanoma [30]. Several studies revealed that tumor lymphangiogenesis promotes enhanced sentinel or regional lymph node metastasis in several types of cancer. Importantly, tumor lymphangiogenesis predicts reduced patient survival in melanoma patients in addition to correlation with enhanced lymph node metastasis [9].

The VEGF-C/VEGFR-3-mediated signaling pathway plays a key role in tumor lymphangiogenesis [3,4,31]. Experimental tumor models showed that VEGFR-3 blockade by neutralizing antibodies or by a soluble form of VEGFR-3 markedly reduced VEGF-C-induced tumor lymphangiogenesis and subsequent draining lymph node metastasis [32–34]. Furthermore, phage display techniques revealed that a monoclonal single chain fragment targeting human VEGF-C inhibits its binding to VEGFR-2/KDR as well as to VEGFR-3/Flt4 [35].

Pathological lymphangiogenesis is induced by several growth factors such as VEGF-A in addition to VEGF-C. Targeted overexpression of VEGF-A in mouse skin promotes tumor-associated angiogenesis within primary sites in a chemically-induced skin carcinogenesis model, leading to an accelerated formation of leaky tumor blood vessels and of squamous cell carcinomas [6] (Fig. 4). The targeted overexpression of VEGF-A as well as VEGF-C also promoted enhanced tumor lymphangiogenesis and draining lymph node metastasis in experimental animal models [6,7]. More importantly, VEGF-A and VEGF-C-overexpressing tumors induced new lymphatic vessel growth in draining lymph nodes even before the tumors metastasized [6,7]. Lymph node lymphangiogenesis likely contributes to tumor metastasis to distant lymph nodes and beyond in these experimental models. Recent studies also showed that lymph node lymphangiogenesis correlated with metastases in distant lymph nodes or distant organs in several types of cancer [10,36,37].

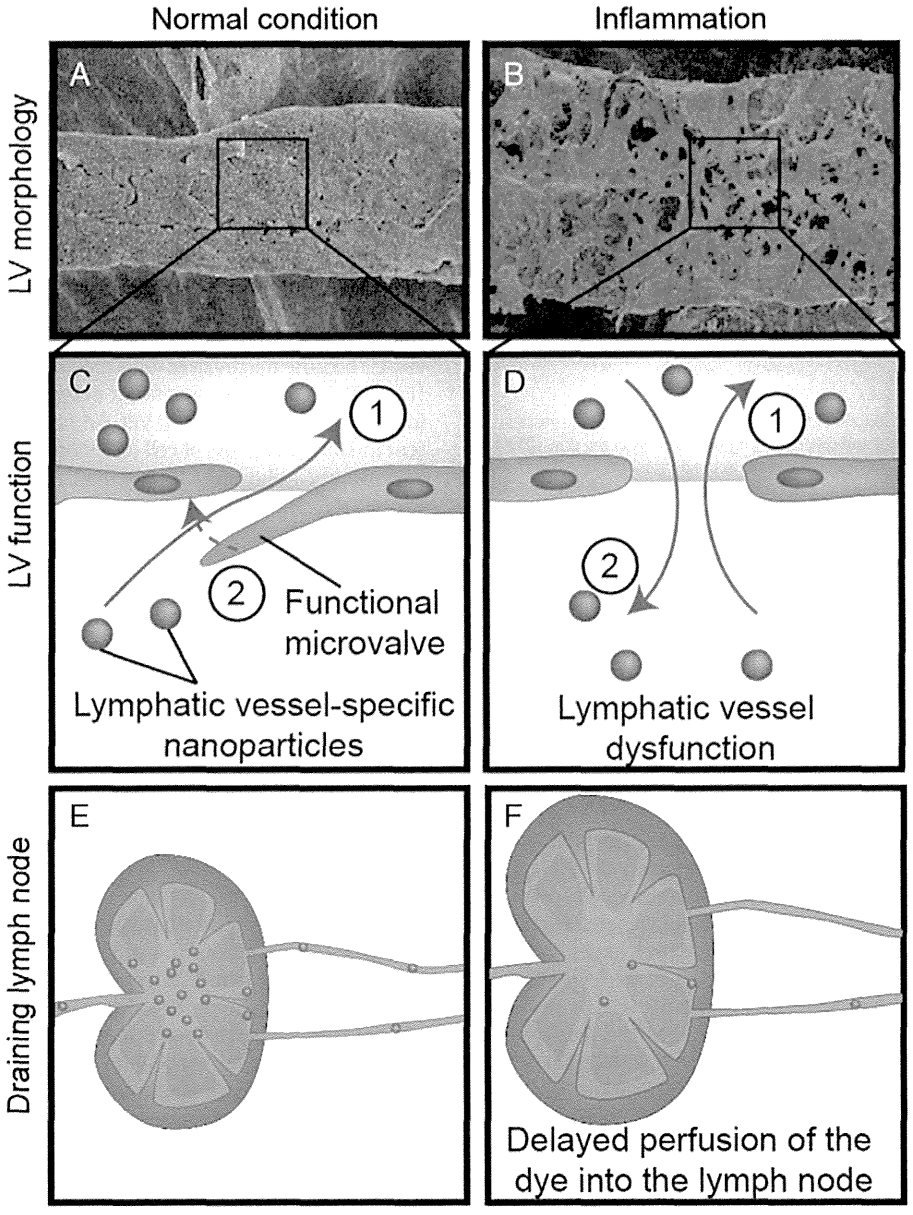

Fig. 3. Impaired lymphatic flow in pathological lymphangiogenesis. (A, B) Scanning electron microscopy demonstrates that the initial cutaneous lymphatic vessels form button-like junctions with overlaps by lymphatic endothelial cells in the normal skin (A). Targeted overexpression of VEGF-A in mouse skin induced chronic inflammation, led to a marked decrease of cell adhesion by lymphatic endothelial cells and the formation of fenestration in the initial lymphatic vessels (B). (C, D) Schematic images of insets from the scanning electron microscopy analysis. In normal skin, initial lymphatic vessels are capable of absorbing nano-particles from the interstitial space (1) into the lymphatic lumen because microvalves contribute to the physiological function (C). In contrast, lymphatic vessels in chronic inflammation lack functional microvalves and show impaired absorption and transport of nanoparticles (D). (E, F) Nanoparticles are efficiently transported by lymphatic vessels from uninflamed skin to the draining lymph nodes, whereas lymphatic nanoparticle transport to lymph nodes is delayed in inflammation.

Lymphatic vessels are collapsed under physiological conditions. Furthermore, tumor-associated lymphatic vessels are exposed to increased interstitial pressure in the tumor microenvironment. Therefore, it remains controversial whether tumor-associated lymphatic vessels are functional. Recent studies in B16 melanoma-bearing mice showed that these vessels take up PEGylated fluorescent dyes and efficiently transport the tracer towards collecting lymphatic vessels [38], indicating that tumor-associated lymphatic vessels are functional with regard to fluid transport. VEGF-C-overexpression in the tumor cells markedly increased lymphatic flow from the primary sites to the draining lymph nodes, and subsequently increased the incidence of tumor metastases [39]. Recent experimental studies showed that VEGF-C also increases the lymphatic flow and clearance of macromolecules in several pathological conditions including acute and chronic inflammation [2,40]. Thus, VEGF-C is one of the crucial factors in promoting lymphatic function as well as lymphatic vessel growth in physiological and pathological settings.

7. Potential role of lymphatic vessels in the enhanced permeability and retention effect

Blood vessels are structurally altered during tumor progression. In experimental tumor models and in certain types of human cancer, tumor-associated blood vessels are fenestrated (Fig. 4). Although these blood vessels are functionally altered, abnormal fenestration and increased plasma leakage represent a potential advantage in drug

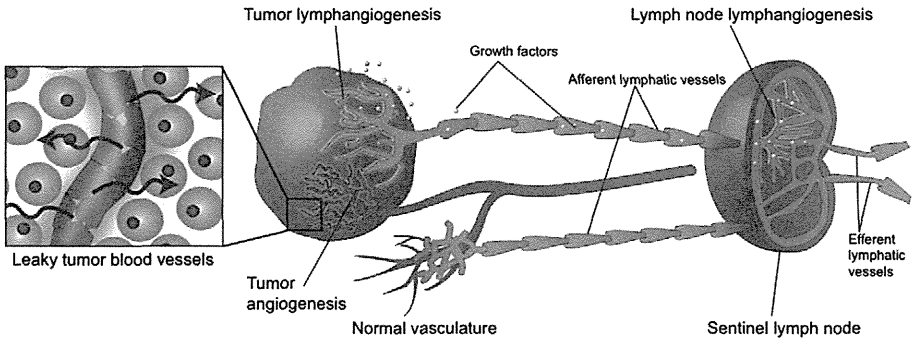

Fig. 4. Tumor and lymph node lymphangiogenesis. Several types of cancer induce tumor-associated lymphatic vessel growth as well as tumor angiogenesis. A subpopulation of newly-formed blood vessels shows increased permeability, leading to an increase of interstitial tissue fluid in the tumor microenvironment. Tumor lymphangiogenesis promotes enhanced sentinel lymph node metastasis, and also mediates the transport of lymphangiogenic growth factors, leading to sentinel lymph node lymphangiogenesis prior to tumor metastasis.

delivery to the tumors and prolonged retention within the specific sites. The enhanced permeability and retention (EPR) effect has promoted the current development of nano-scaled carriers in combination with conventional drugs against cancer [41,42]. It remains to be elucidated whether lymphatic vasculature promotes the EPR effect during drug delivery and excretion in the tumor microenvironment [43]. Physiological lymphatic flow supports the clearance of interstitial tissue fluid and promotes efficient drug delivery to the affected lesions. However, several pathological conditions potentially alter the function of lymphatic vessels. VEGF-A plays a major role in promoting tumor angiogenesis that mediates tumor progression. Importantly, targeted overexpression of VEGF-A in mouse skin led to a marked enlargement and fenestration of lymphatic vessels as well as blood vessels (Fig. 3B), resulting in a reduced lymphatic transport from the skin lesions to the draining lymph nodes [2]. Thus, the maintenance of proper lymphatic morphology and the reconstitution of lymphatic function represent key elements for the efficient drug delivery into the tumor microenvironment and in chronic inflammation.

VEGF-C is one of the crucial growth factors that increase lymphatic flow, potentially contributing to the EPR effect during drug delivery in several diseases such as acute and chronic inflammation. However, VEGF-C overexpression in experimental tumor models showed an accelerated formation of tumor metastases in draining lymph nodes [7,39]. Increased lymphatic flow may lead to more metastasis to draining lymph nodes in the experimental tumor models. Therefore, other therapeutic approaches should be considered for the proper lymphatic flow to mediate efficient drug delivery to tumors. Recent evidence indicates that bevacizumab, a VEGF-A-neutralizing antibody, potentially leads to a recovery of blood flow as well as to inhibition of new blood vessel growth in certain types of cancer, when the neutralizing antibody is combined with conventional chemotherapy [44,45]. Based on the significant influence of VEGF-A on the lymphatic vasculature in experimental tumor models, VEGF-A blockade might also normalize the lymphatic flow in the tumor microenvironment to achieve more efficient drug delivery.

8. Nanophysiology in lymphatic research and biology

Recent advances in biochemistry and photo-engineered technology enabled the generation and visualization of nano-scaled particles for experimental *in vivo* imaging. In particular, near-infrared (NIR) (700–900 nm) fluorescence imaging represents a feasible way to visualize lymphatic vessels *in vivo* in pathological and physiological conditions. ICG is a useful dye for lymphatic imaging. Although the application of ICG-mediated fluorescence depends on non-specific binding to carrier proteins such as albumin, ICG may help to translate experimental research to clinical settings since ICG is the only NIR compound currently approved for human application worldwide. Recent studies showed that liposomal encapsulation of ICG improves the stability and brightness of the fluorescent dye, and further provides specificity for the lymphatic system, as compared to non-capsulated ICG that is also taken up by blood vessels [39].

The advantage of nano-scaled carriers is reflected by the customization of size and shape as crucial parameters. Initial lymphatic vessels are capable of taking up molecules of less than 100 nm in diameter under physiological conditions [46]. In contrast, lymphatic vessels in certain pathological conditions may develop fenestrations, indicating that these lymphatic vessels are leaky, and less efficient in the transport of the biochemical particles. Therefore, the quantitative analysis of tracers with specific diameter and shape might help to assess the biological function of lymphatic vessels in physiological and pathological settings such as chronic inflammation and tumor progression.

9. Perspectives

In the 17th century, Gasparo Aselli discovered the "milky veins", which represented the lacteal flow in the mesentery of a dog [47]. Since then, the visualization of lymphatic vessels has enabled a better understanding of the dynamics of lymphatic flow and of the function of lymphatic vessels at the nano-scale level. To date, the biological significance of lymphatic vessels is relatively well understood in cancer biology, leading to new therapeutic approaches. So far, studies of lymphatic biology have clarified the molecular mechanisms of lymphatic vessel development in physiological and pathological settings. Although lymphatic endothelial cells represent the major focus of investigations regarding the function of lymphatic vessels [48–50], these vessels are composed of several other cell types such as pericytes and smooth muscle cells, and of extracellular matrix molecules, which are susceptible to pathological settings and might directly influence the drug excretion. Therefore, strategies for efficient drug delivery based on lymphatic biology require further detailed assessments of the cellular interactions between lymphatic endothelial cells and other cell types under physiological and pathological conditions.

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ORIGINAL ARTICLE

Role of laparoscopy and ultrasound in the management of "impalpable testis" in children

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KEYWORDS
undescended testis;
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Summary *Background:* Undescended testes is one of the most common congenital abnormalities in boys. In cases of impalpable testes, ultrasound is often used to find the testis, which frequently provides false-negative results. Recently, laparoscopy has become popular in the management of impalpable testes.
Methods: Retrospective study of all children with impalpable testes presenting for laparoscopy between August 2007 and July 2011 who had undergone ultrasound examinations without localizing the testes was conducted and the role of laparoscopy in diagnosing impalpable testes was evaluated.
Results: Twenty-three patients presented with impalpable testes for laparoscopy. All patients underwent ultrasound examinations in which the testes could not be identified. Of the 23 patients, Five patients were found to have palpable testes in the superficial inguinal pouch under anesthesia and proceeded to conventional open exploration during which the testes were brought into the scrotum. Eighteen patients were found to have impalpable testes in an evaluation under anesthesia (EUA) and proceeded to laparoscopy. Twelve patients were found to have intra-abdominal testes and underwent laparoscopic-assisted orchidopexy. Three patients

Conflicts of interest: The authors declare that they have no financial or non-financial conflicts of interest related to the subject matter or materials discussed in the manuscript.

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underwent a two-stage Fowler-Stephens procedure, and two patients with "vanishing" testes with the vas and atrophic vessels entering a closed internal ring proceeded to open exploration and orchidectomy for atrophic testes. In addition, a teenager with atrophic testes underwent laparoscopic orchidectomy.

Conclusions: Laparoscopy is superior to ultrasound in the management of impalpable testes when high-resolution ultrasound is not available during the diagnostic process, with respect to both the sensitivity of localizing the testis and being more time and cost effective.

1. Introduction

Undescended testes in boys is a very common congenital abnormality in which one or both testes do not reach the bottom of the scrotum prior to birth. The incidence of the condition is 3–5% among all boys at birth, and decreases to 0.8–1% after 6 months of age.[1–12] The classification of undescended testes includes suprascrotal, intracanalicular, intra-abdominal, and ectopic types,[6,13] whereas the classification of impalpable testes includes intra-abdominal, intracanalicular, vanishing testis, and agenesis testis types.[14] The diagnostic tools used to detect undescended testes include physical examinations, radiologic examinations, hormonal examinations, and laparoscopic examinations.[1,4,10,15–18] When the testes cannot be detected by physical examination, ultrasound is the most favored tool among the radiologic examinations because it is the least invasive procedure and does not scare children, whereas other radiologic examinations require anesthetic procedures and the associated risk of radiation. Ultrasound is also a fast and simple procedure to perform. Despite its advantages, ultrasound provides the lowest sensitivity results in localizing impalpable testes,[2,4,6,8,18–20] unless high-resolution ultrasound is used, which achieves similar outcomes as computed tomography scans.[1,6] However, high-resolution ultrasound is not widely available in most centers in developing countries. The lack of equipment sometimes leads to misdiagnosis and misleads the parents into not taking further action and assuming that the testes had not formed.

Males with undescended testes have a lower sperm count, poorer quality sperm, and lower fertility rate, compared to males whose testicles descend normally; the rate of subfertility increases with bilateral involvement and increasing age at the time of orchidopexy.[1,4] Based on the results of testicular biopsies performed at the time of orchidopexy, the germ cell density decreases over time, beginning as early as 1 year of age.[4] Fertility is directly related to the age at orchidopexy with a success rate as high as 87.5% if the surgery is performed before 2 years of age and as low as 14% if the surgery is performed at 13 years of age or older; however, these figures may be influenced by the distribution of unilateral and bilateral cases.[2]

Undescended testes are not a life-threatening disease. Therefore, most parents try to avoid any invasive procedures they believe are unnecessary. However, the long-term risk of testicular cancer has been well documented in males with a history of undescended testes, particularly if surgical correction is performed after 1 year of age.[1,2,4,6–8,11,12] Hence, making a correct diagnosis is essential to ensure that the patient receives proper treatment at the appropriate time to reduce the risk of testicular cancer.

Laparoscopy has become widely used to diagnose impalpable testes since 1976 when Cortesi et al[15] initially described it. Using laparoscopy, the testes can be clearly identified and localized, and treatment can be administered immediately after making a diagnosis. Therefore, the risk of misdiagnosing agenesis testis in impalpable testes is reduced, which then reduces the risk of testicular cancer over the long term.

2. Patients and methods

The diagnosis of impalpable testis in patients who were referred to Universiti Kebangsaan Malaysia (UKM) Hospital Pediatric Surgery Outpatient Clinic in Kuala Lumpur, Malaysia between August 2007 and July 2011 was confirmed by physical examinations (Fig. 1). Based on the UKM Clinical Pathway Guidelines, the patients were eligible for an evaluation under anesthesia and proceeded to open orchidopexy if the testes were palpable, or they underwent diagnostic laparoscopy and then proceeded to the appropriate surgery. If intra-abdominal testes were detected, the testes were evaluated with respect to whether they could be brought down into the scrotum without tension by bringing the testis to the contralateral side. If there was no tension, the procedure was continued with laparoscopic-assisted orchidopexy. If there was tension, the procedure was continued with the two-stage Fowler–Stephens technique.[1] If remnants of the testes were present or if the vas deferens and vessels entered the inguinal canal, the procedure was continued with exploration and orchidectomy of the atrophic testis. If the testes were found to be atrophic intra-abdominally, a laparoscopic orchidectomy was performed.

3. Results

Prior to being referred to the UKM Hospital Pediatric Surgery Outpatient Department, 23 patients with impalpable testes presented for laparoscopy after having undergone ultrasound. No testes were identified on ultrasound examinations. Five patients had palpable testes in the superficial inguinal pouch under anesthesia, and proceeded to conventional open exploration during which the testes were brought into the scrotum without tension. Eighteen (18/23)

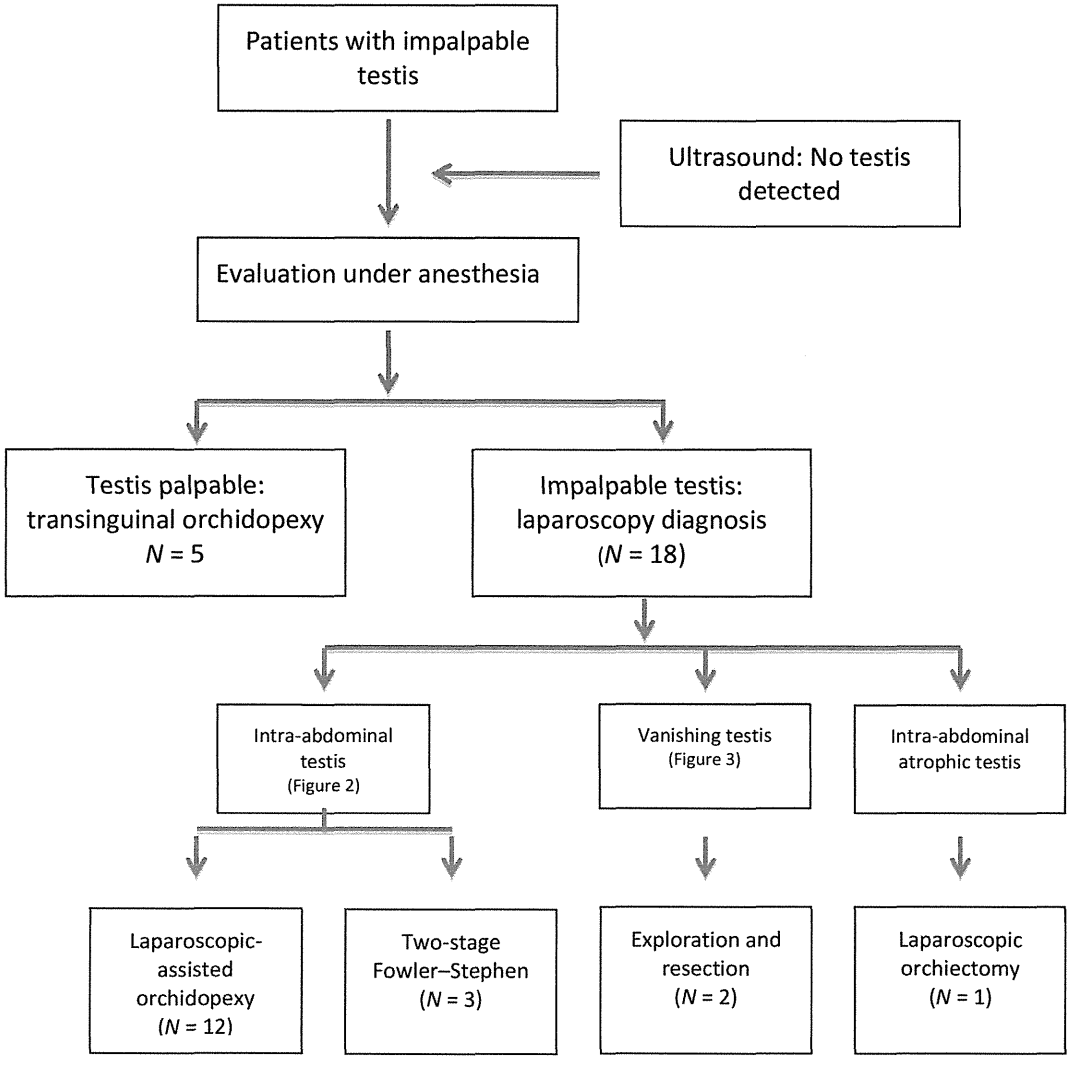

Figure 1 Impalpable testis management algorithm used at Universiti Kebangsaan Malaysia Hospital (Kuala Lumpur, Malaysia) and the number of patients treated between August 2007 and December 2011.

patients had impalpable testes in an evaluation under anesthesia (EUA) and proceeded to laparoscopy. Twelve patients had intra-abdominal testes and underwent laparoscopic-assisted orchidopexy (Fig. 2). Three patients underwent the two-stage Fowler–Stephens procedure. Two patients with vanishing testes with the vas deferens and atrophic vessels entering a closed internal ring proceeded to open exploration and orchidectomy for atrophic testes (Fig. 3). In addition, a teenager with atrophic testes underwent laparoscopic orchidectomy.

4. Discussion

Failure to locate the testes, interpreting this finding as indicating that no testes had formed, and diagnosing the patient with agenesis testis is a relief to some parents as this diagnosis has no operative risks and no cancer risks because of undescended testes, and saves money due to the lack of the need for surgery. However, should a misdiagnosis occur, and the testes actually exists somewhere in the abdomen, then the future risk of testicular cancer and infertility must be considered. Therefore, meticulous examinations using sensitive and detailed diagnostic tools should be conducted to detect the testes or remnants and to prevent the risk of testicular cancer in the future.

Figure 2 Intra-abdominal testis. The testis is located at the outer ring of the inguinal canal.

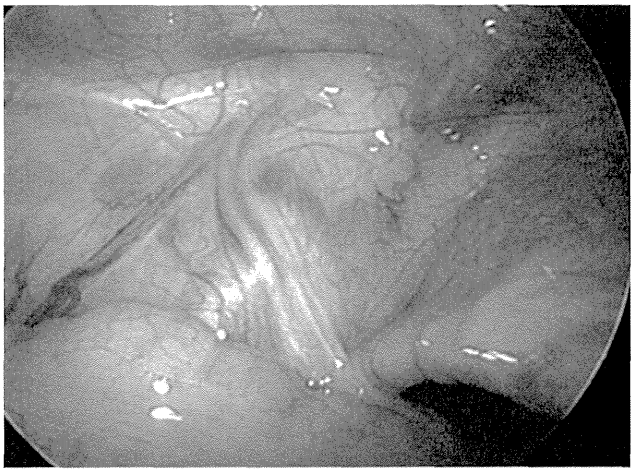

Figure 3 Vanishing testis. The vas deferens and vessels enter the inguinal canal.

Ultrasound is an operator-dependent diagnostic tool that provides insensitive results, as many studies prove.[4,8,18–20] High-resolution ultrasound offers similar sensitivity as computed tomography, although this modality is unavailable in most centers in developing countries. The experience of the ultrasound operator has an important role in the efficacy of the ultrasound examination. Magnetic resonance imaging is more sensitive than ultrasound and does not have the risk of radiation or require intravascular contrast injection; however, it has disadvantages such as a long scanning time and the presence of motion artifacts during the examination.[20]

Laparoscopy has become widely used to diagnose impalpable testes since 1976 when Cortesi et al[15] first reported its use. Laparoscopy provides the ability to visualize directly the testes or remnants, detect agenesis of the testes, and evaluate the possibility of bringing the testes into the scrotum without tension. If no testes are present, tracking to the distal part of the vas deferens and the vascular area is possible using direct visualization with the laparoscopy camera to detect whether the testes are atrophic, contain remnants, or simply do not exist. Therefore, a diagnosis can be determined without hesitation.

If the testes are detected on ultrasound examination, the patient must be considered for surgery. The delay between diagnosis and surgery may allow the parents to change their minds regarding the operation, thereby increasing the patient's risk of testicular cancer in the future. This is a common situation because the parents believe that there is no danger because the child appears healthy and is not in a life-threatening situation.

If the testes are not detected on ultrasound, the patient must undergo an evaluation under anesthesia to obtain greater relaxation of the muscles, thereby making palpation more effective.[17] An evaluation under anesthesia is performed in an operating theater. Therefore, if the testes are palpable, open orchidopexy can be performed immediately. If the testes are not palpable, the laparoscopic diagnostic procedure is continued with the appropriate procedure, depending on the results obtained from the examination. For example, the patient may receive laparoscopic-assisted orchidopexy, laparoscopic orchidectomy, the Fowler–Stephens procedure, or exploration and excision of the remnants of the testes. The operation may be terminated because of findings of agenesis testis.

Whether surgery is performed, patients can be diagnosed in one stage using diagnostic laparoscopic procedures, which makes the procedure more time- and cost-effective. For these reasons, laparoscopy is superior to ultrasound.

This study showed that laparoscopy can be used to find testes that are not detected on ultrasound. It addresses the problems associated with impalpable testes in one stage. Our results support the findings of Elder[19] who concluded: "ultrasonography is unnecessary in boys with a nonpalpable testis, because it rarely if ever localizes a true nonpalpable testis, and it does not alter the surgical approach in these patients." In a 2011 review article, Tasian and Copp[9] also concluded: "ultrasound does not reliably localize nonpalpable testis and does not rule out an intra-abdominal testis. Eliminating the use of ultrasound will not change the management of non-palpable cryptorchidism but will decrease health care expenditures."

With respect to the sensitivity of localizing the testis and being more time- and cost-effective, laparoscopy is superior to ultrasound in the management of impalpable testes when high-resolution ultrasound is unavailable during the diagnostic process.

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ORIGINAL ARTICLE

Therapeutic potential of mesenchymal stem cell transplantation in a nitrofen-induced congenital diaphragmatic hernia rat model

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Abstract
Purpose The aim of this study was to evaluate the efficacy of mesenchymal stem cells (MSCs) in a nitrofen-induced congenital diaphragmatic hernia (CDH) rat model.
Methods Pregnant rats were exposed to nitrofen on embryonic day 9.5 (E9.5). MSCs were isolated from the enhanced green fluorescent protein (eGFP) transgenic rat lungs. The MSCs were transplanted into the nitrofen-induced E12.5 rats via the uterine vein, and the E21 lung explants were harvested. The study animals were divided into three: the control group, the nitrofen-induced left CDH (CDH group), and the MSC-treated nitrofen-induced left CDH (MSC-treated CDH group). The specimens were morphologically analyzed using HE and immunohistochemical staining with proliferating cell nuclear antigen (PCNA), surfactant protein-C (SP-C), and α-smooth muscle actin.
Results The alveolar and medial walls of the pulmonary arteries were significantly thinner in the MSC-treated CDH group than in the CDH group. The alveolar air space areas were larger, while PCNA and the SP-C positive cells were significantly higher in the MSC-treated CDH group, than in the CDH group. MSC engraftment was identified on immunohistochemical staining of the GFP in the MSC-treated CDH group.
Conclusions MSC transplantation potentially promotes alveolar and pulmonary artery development, thereby reducing the severity of pulmonary hypoplasia.

Keywords Mesenchymal stem cells · Congenital diaphragmatic hernia · Pulmonary hypoplasia · Pulmonary hypertension

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Introduction

According to recent advances in postnatal therapy, there have been several reports of improvements in the treatment outcomes of congenital diaphragmatic hernia (CDH) [1, 2]. However, CDH patients with severe pulmonary hypoplasia continue to exhibit high morbidity and mortality. Pulmonary hypoplasia, defined as arrest in lung development, is characterized by decreased airway branching, thickened alveolar walls, increased interstitial tissue and decreased alveolar air space [3, 4]. In experimental animal models of CDH, several treatments, such as the prenatal administration of growth factor, vitamin E and retinoid acid, have suggested to improve lung hypoplasia [4–6]. However, despite the introduction of new drugs and changes in management, limitations persist, and these therapies appear to be far from being able to truly cure lung hypoplasia.

Currently, the efficacy of a type of prenatal intervention, named percutaneous fetoscopic endoluminal tracheal occlusion (FETO) therapy, is being investigated in the tracheal occlusion to accelerate lung growth trial [(TOTAL); a European and North American collaboration]

[7]. The use of FETO remains controversial, as some papers have reported improvements in the survival of patients with severe CDH, although neonates and infants treated with this therapy subsequently display obvious tracheomegaly [7–9]. Therefore, it may be necessary to combine additional experimental therapies, such as cellular therapy and tissue engineering approaches, to reduce the risk of harmful complications [7, 10].

Mesenchymal stem cells (MSCs) are widely used in experimental research as a source of cell-based therapy. MSCs are known to be self-renewing, with the ability to form an adherent cell layer in a plastic standard culture dish and exhibit a combination of phenotypic and functional characteristics [11]. MSCs can be isolated from the stromal tissues of various adult organs, including bone marrow, muscle, amniotic fluid, adipose tissue and the dermis and lungs [11, 12]. MSCs also display specific characteristics, which are able to self-renew and differentiate into osteocytes, chondrocytes, adipocytes, myofibroblasts, and smooth muscle cells (multipotent differentiation) [12]. Other characteristics of MSCs include their fibroblast-like shape in culture, extensive capacity for proliferation and negative for hematopoietic stem cells (HSCs) and endothelial cell surface marker [10–13]. Their tendency to exhibit low immunogenicity may also make them appropriate for use in allogeneic transplantation [10, 13].

A number of studies have demonstrated the efficacy of MSC transplantation in treating pulmonary diseases, such as that observed in bleomycin, endotoxin, and lipopolysaccharide (LPS)-induced lung injury models [10, 14]. Aslam et al. [14] proposed that the application of bone marrow MSCs and their secreted factors offers the potential for new therapeutic approaches in cases of neonatal chronic lung disease.In addition, cellular therapies have been suggested to have favorable effects in improving serious pediatric lung diseases such as pulmonary hypoplasia and cystic fibrosis [7, 10, 15]. However, the efficacy and mechanisms of MSC transplantation in CDH models have not been fully clarified.

The aim of this study was, therefore, to evaluate the efficacy of MSC therapy in a nitrofen-induced CDH rat model, based on the detection of lung maturation and a reduction in the degree of pulmonary hypertension.

Materials and methods

Experimental animals

The animal experiments were approved by the Institutional Animal Care and Use Committee of Kyushu University (approval no. A-25-175-0). Pregnant Wistar rats were purchased from a commercial breeder (Japan Kyudo, Inc., Saga, Japan) and randomly divided into three groups: the control group ($n = 6$), the nitrofen-induced left CDH group (CDH group, $n = 5$), and the MSC-treated nitrofen-induced left CDH group (MSC-treated CDH group, $n = 6$). Pregnant rats in both the CDH and MSC-treated CDH groups were exposed intragastrically to 100 mg of nitrofen (2,4-dichlorophenyl-*p*-nitrophenyl ether, Wako, Japan), dissolved in olive oil on embryonic day 9.5 of gestation (E9.5), whereas those in the control group received vehicle only [4].

Isolation and culture of MSCs

Mesenchymal stem cells were isolated from the lungs of donor adult enhanced green fluorescent protein (eGFP) transgenic SD rats [SD-Tg(CAG-EGFP), Japan SLC Inc., Shizuoka, Japan]. The harvested lung tissues were flushed with phosphate-buffered saline (PBS) to wash out the blood, and the attached trachea and connective tissue were subsequently removed. The tissues were then minced, and treated with PBS containing 0.4 % collagenase type I (Worthington Biochemicals, Lakewood, NJ, USA) and 0.3 % dispase II (Sanko Junyaku, Tokyo, Japan) for 1 h at 37 °C. The digested samples were filtered using a 70-μm cell strainer (BD Bioscience, San Jones, CA, USA) to obtain a single-cell suspension. The cells were seeded at 1×10^6 cells in a 75-cm^2 tissue culture flask and incubated at 37 °C with 5 % CO_2. Twenty-four hours after incubation, non-adherent cells were removed by washing twice with PBS, and the adherent cells were cultured with a growth medium. The growth medium consisted of 20 % fetal bovine serum (FBS) (Equitech-Bio, Kerrville, TX, USA), 100 μM of L-ascorbic acid 2-phosphate (Wako Pure Chemical, Osaka, Japan), 2 mM L-glutamine (Nacalai Tesque, Kyoto, Japan), an antibiotic mixture containing 100 U/ml of penicillin and 100 μg/ml of streptomycin (Nacalai Tesque), and 250 ng/ml amphotericin B (Fungizone, Life technologies, USA) in alpha minimum essential medium (αMEM, Invitrogen, Grand Island, NY, USA). After reaching confluence, the adherent cells were harvested using 0.25 % trypsin and 0.1 mM EDTA solution, and reseeded at a density of 0.2×10^6 cells in 100-mm tissue culture dishes. The passaging of the cultured cells was repeated an additional two or four times to generate a sufficient number of cells for transplantation.

MSC transplantation

On E12.5, MSCs isolated from the lung tissues of the eGFP rats were transplanted into the fetuses. Under deep pentobarbital anesthesia (50 mg/kg, i.p.), MSCs suspended in PBS were intravenously injected via the uterine vein in the bilateral horn of the uterus of the dams, with a total of