Figure 2. Bar plots of the number of exacerbation events in each month during the follow-up period

Exacerbation was defined by the symptom definition, prescription definition, antibiotic definition, and admission definition.

Figure 2

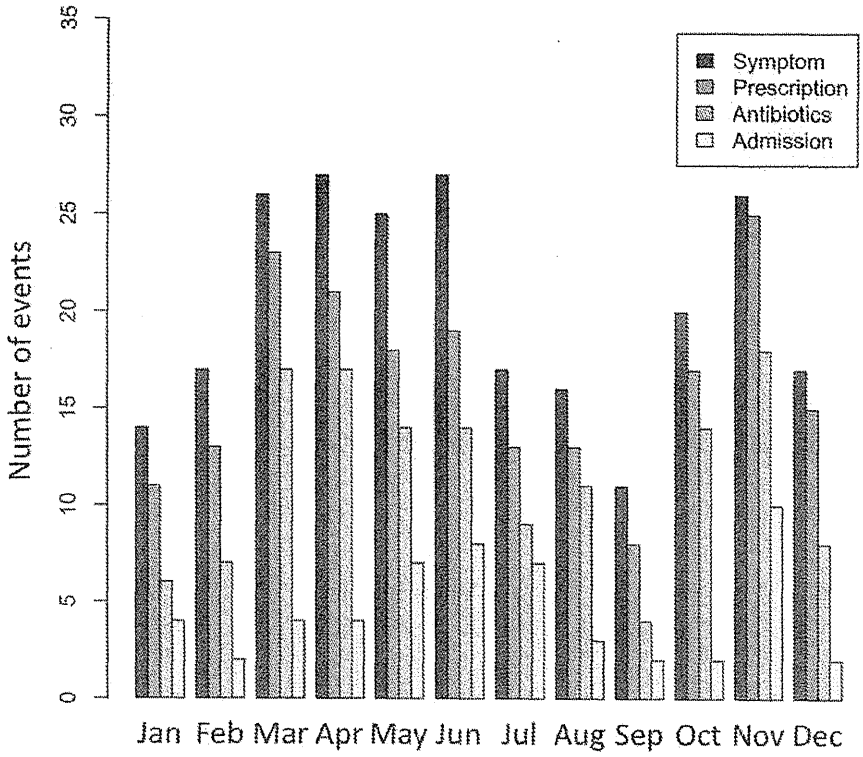

Number of events
0
5
10
15
20
25
30
35
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Symptom
Prescription
Antibiotics
Admission

Figure 3. Box plots of the exacerbation frequency after the first year of follow-up

Subjects were devided into two groups: subjects who did not experience exacerbations within the first year of follow-up (Event ≤1y (-)) and subjects who experienced exacerbations within the first year of follow-up (Event ≤1y (+)). Graphs show exacerbation defined by the symptom definition, the prescription definition, the antibiotic definition, and the admission definition, respectively.

Figure 3

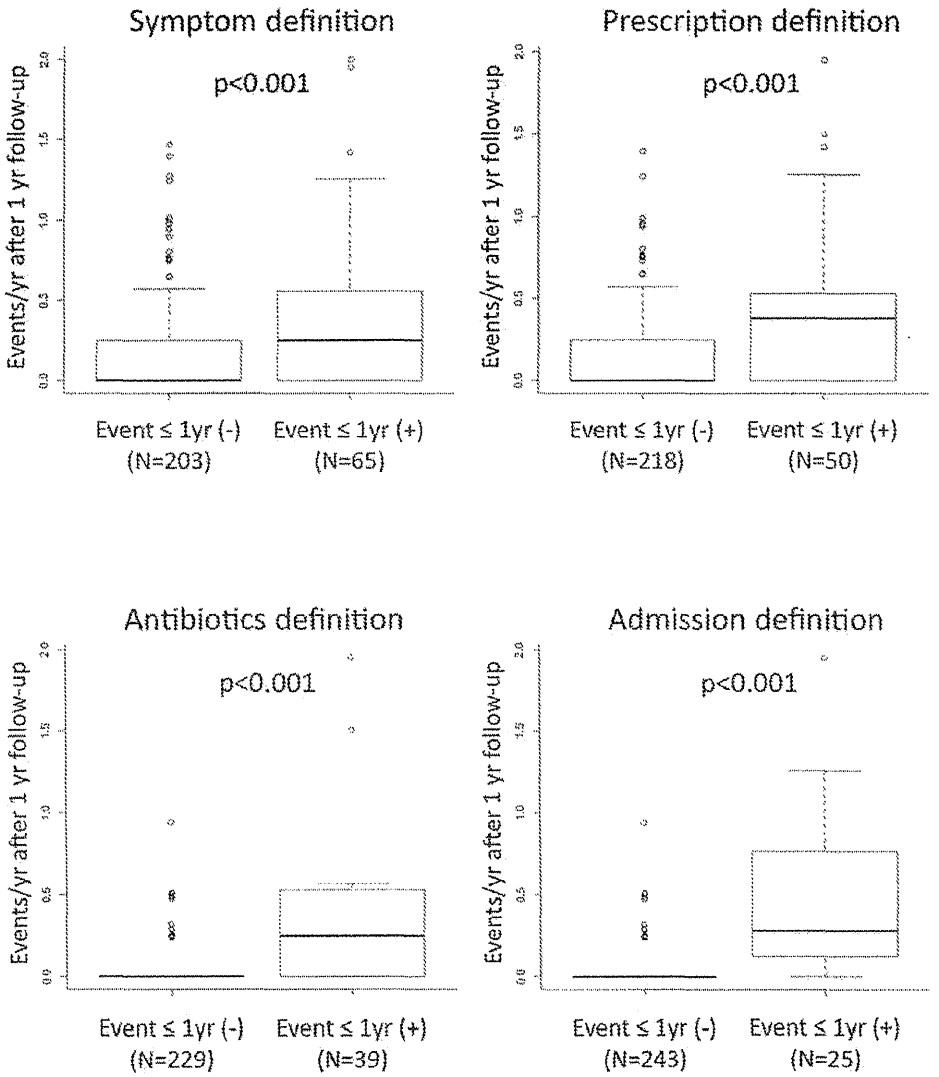
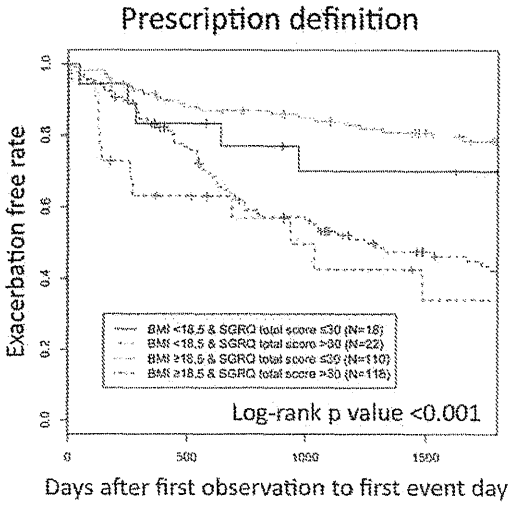
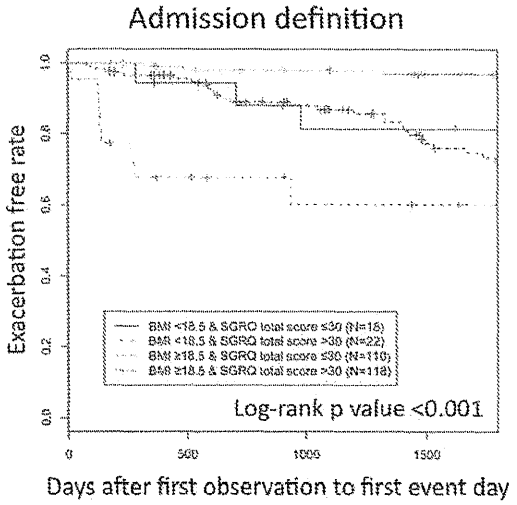

Symptom definition
p<0.001
Events/yr after 1 yr follow-up
Event ≤ 1yr (-)
(N=203)
Event ≤ 1yr (+)
(N=65)
Prescription definition
p<0.001
Events/yr after 1 yr follow-up
Event ≤ 1yr (-)
(N=218)
Event ≤ 1yr (+)
(N=50)
Antibiotics definition
p<0.001
Events/yr after 1 yr follow-up
Event ≤ 1yr (-)
(N=229)
Event ≤ 1yr (+)
(N=39)
Admission definition
p<0.001
Events/yr after 1 yr follow-up
Event ≤ 1yr (-)
(N=243)
Event ≤ 1yr (+)
(N=25)

Figure 4. Kaplan-meier curves for exacerbation-free survival

Subjects were devided into four groups according to SGRQ total score and BMI value. Graphs showing exacerbation defined by the prescription definition and the admission definition are shown.

Figure 4


Prescription definition
Exacerbation free rate
0.0
0.2
0.4
0.6
0.8
1.0
0
500
1000
1500
BMI <18.5 & SGRQ total score ≤30 (N=18)
BMI <18.5 & SGRQ total score >30 (N=22)
BMI ≥18.5 & SGRQ total score ≤30 (N=110)
BMI ≥18.5 & SGRQ total score >30 (N=118)
Log-rank p value <0.001
Days after first observation to first event day


Admission definition
Exacerbation free rate
0.0
0.2
0.4
0.6
0.8
1.0
0
500
1000
1500
BMI <18.5 & SGRQ total score ≤30 (N=18)
BMI <18.5 & SGRQ total score >30 (N=22)
BMI ≥18.5 & SGRQ total score ≤30 (N=110)
BMI ≥18.5 & SGRQ total score >30 (N=118)
Log-rank p value <0.001
Days after first observation to first event day

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Histology and Histopathology
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Review

Crosstalk between endothelial cell and thrombus in chronic thromboembolic pulmonary hypertension: perspective

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Summary. It is generally accepted that chronic thromboembolic pulmonary hypertension (CTEPH) results from pulmonary emboli originating from deep vein thrombosis. However, this consensus opinion has been challenged, and the concept that some aspects of CTEPH exacerbation might result from a small-vessel disease leading to secondary thrombosis has been suggested.

In addition to the effect of recurrent thromboembolism, a number of lines of clinical evidence indicate that progressive worsening is contributed to by remodeling in the small pulmonary arteries. Histopathological studies of the microvascular changes in CTEPH have identified vascular lesions similar to those seen in idiopathic pulmonary arterial hypertension (IPAH). Especially in *in vitro* and *ex vivo* experiments, pulmonary artery endothelial cells (ECs) in pulmonary hypertensive diseases are suggested to exhibit an unusual hyperproliferative potential with decreased susceptibility to apoptosis, indicating that dysfunctional ECs may contribute to the progression of the diseases. Although the degree and mechanisms of EC dysfunction as a contributor to CTEPH are unclear, EC dysfunction may occur in small arteries. Indeed, the cells stimulated by the microenvironment created by the unresolved clot may release substances that induce EC dysfunction. The EC dysfunctions in CTEPH may lead to disorders of the anti-coagulation properties in ECs and may result in additional clots *in situ*. Moreover, these may lead to the progression, not only of distal thrombus, but also of proximal clotting.

This article reviews the pathobiological concepts of CTEPH and explains a crosstalk between EC dysfunction and *in situ* thrombi which may contribute to the vascular lesions of CTEPH.

Key words: Endothelial cell, Thrombus, CTEPH

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Introduction

Chronic thromboembolic pulmonary hypertension (CTEPH) has emerged as one of the leading causes of severe pulmonary hypertension. CTEPH is characterized by intraluminal thrombus formation and fibrous stenosis or complete obliteration of the pulmonary arteries (Klepetko et al., 2004). The consequence is increased pulmonary vascular resistance, resulting in pulmonary hypertension and progressive right heart failure. Pulmonary endarterectomy (PEA) is the current mainstay of therapy for CTEPH (Jamieson et al., 2003). Recently, there has been evidence suggesting that the existing consensus that the pathophysiology of CTEPH results from unresolved pulmonary emboli may have been too simplistic (Hoeper et al., 2006). Although acute pulmonary embolism is generally accepted as the main initiating event in CTEPH, small-vessel disease is believed to appear and worsen later during the course of disease, and to contribute to the progression of hemodynamic and symptomatic decline (Hoeper et al., 2006). Moreover, *in situ* thrombosis and pulmonary arteriopathy have been proposed as potential causes of CTEPH (Shure, 1996; Peacock et al., 2006).

This article reviews the pathobiological concepts of CTEPH, including pulmonary microvascular disease, the endothelial-mesenchymal transition (EnMT), EC dysfunction, and *in situ* thrombosis, which are important pathological features of pulmonary arterial hypertension (PAH) (Eisenberg et al., 1990; Welsh et al., 1996; Wolf et al., 2000; Bauer et al., 2002; Cool et al., 2004; Humbert et al., 2004; Reesink et al., 2004). Furthermore,

it explains a crosstalk between EC dysfunction and *in situ* thrombi which may contribute to the vascular lesions of CTEPH.

Microvascular lesions

In addition to the effect of recurrent thromboembolism, a number of lines of clinical evidence indicate that progressive worsening is contributed to by remodeling in the small distal pulmonary arteries in the open vascular bed (Moser and Bloor, 1993; Azarian et al., 1997; Yi et al., 2000). Indeed, the PH and right ventricular dysfunction are progressive, even in the absence of recurrent thromboemboli (Azarian et al., 1997). Moreover, there is a low degree of correlation between the extent of vascular obstruction visible on pulmonary angiography and the severity of PH (Azarian et al., 1997). There is likely a vascular stealing phenomenon, which means that there is redistribution of the pulmonary blood flow from the nonoccluded to newly endarterectomized vasculature after PEA (Moser and Bloor, 1993). There is often no hemodynamic improvement and persistent PH despite successful PEA in approximately 35% of patients (Condliffe et al., 2008).

Pulmonary microvascular disease, which is an important pathological feature of PAH, leads to increased pulmonary vascular resistance and reduced compliance, with marked proliferation of pulmonary artery smooth muscle cells (SMCs) and endothelial cells (ECs), resulting in the obstruction of blood flow in pulmonary arteries (Humbert et al., 2004). Recently, we reviewed pathogenetic concepts of pulmonary arterial hypertension (PAH) and explained the vascular lesions with EC dysfunction, i.e., apoptosis and proliferation (Sakao et al., 2009, 2010). Taraseviciene-Stewart et al. showed that a vascular endothelial growth factor (VEGF) receptor blocker induced some of the "angioproliferative" features typical of advanced PAH in a rat model, i.e., worsening of the pathological vascular remodeling, and those features were reversed by inhibitors of apoptosis, suggesting that increased apoptosis of ECs in response to loss of survival signaling provided a selection pressure that induced the emergence of actively proliferating ECs without evidence of apoptosis (Taraseviciene-Stewart et al., 2001). Moreover, our *in vitro* experiments have demonstrated that the emergence of apoptosis-resistant proliferating ECs depended on initial EC apoptosis induced by blockade of VEGF receptors and these phenotypically altered ECs expressed the tumor marker survivin and the antiapoptotic protein Bcl-$_{XL}$ (Sakao et al., 2005). Consistent with our results, Masri et al. have shown that pulmonary artery ECs isolated from patients with idiopathic PAH (IPAH) were hyperproliferative and apoptosis-resistant (Masri et al., 2007). However, these results were from an animal model and tissue culture experiments, not from human. It remains unknown whether they actually contribute to pathobiology of human PAH.

The studies of the microvascular changes in CTEPH have identified histopathological characteristics similar to those seen in IPAH and Eisenmenger's syndrome (Moser and Bloor, 1993; Azarian et al., 1997; Yi et al., 2000; Piazza and Goldhaber, 2011). Therefore, dysfunctional ECs may contribute to the progression of the microvascular changes in CTEPH as shown in PAH. Although PEA is the current mainstay of therapy for CTEPH, a recent study showed that specific vasodilative compounds, e.g., prostanoids, endothelin receptor antagonists, phosphodiesterase type 5 inhibitors or a combination, as used for PAH therapy, improved cumulative survival in the patients with inoperable CTEPH, suggesting that there may be vasodilative reactivity in the vasculature of some populations of CTEPH patients as shown in the vasculature of PAH (Seyfarth et al., 2010). Indeed, there exists evidence that patients with CTEPH show similar acute vasoreactivity to inhaled nitric oxide and iloprost (Ulrich et al., 2006; Skoro-Sajer et al., 2009).

The similarities between the microvascular changes in CTEPH and those seen in IPAH suggest that specific vasodilative compounds as used for PAH therapy may be appropriate for some populations of CTEPH, as the patients with no hemodynamic improvement and persistent PH despite successful PEA.

Endothelial-mesenchymal transition (EnMT)

EnMT is a term which has been used to describe the process through which ECs lose their endothelial characteristics and gain the expression of other mesenchymal cell characteristics (Arciniegas et al., 2007). There is the intriguing possibility that intimal SMCs may arise from ECs (Majesky and Schwartz, 1997). In the systemic circulation, Arciniegas et al. demonstrated that mesenchymal cells that existed in the intimal thickening may arise from ECs (Arciniegas et al., 2000). Indeed, the existence of "transitional cells" demonstrating features of both ECs and vascular SMCs in the plexiform lesions in the lungs from patients with IPAH has been identified (Cool et al., 2004). Our *in vitro* studies of human pulmonary microvascular endothelial cells (HPMVECs) showed that blockade of VEGF receptors generated a selection pressure that killed some ECs and expanded resident progenitor-like cells to transdifferentiate into other mesenchymal phenotypes (Sakao et al., 2007). Although there is the limitation of this study based on *in vitro* experiment, this result may support the concept that transdifferentiation of pulmonary ECs to other mesenchymal cells may contribute to the muscularization of the pulmonary arteries. Because of histopathological similarity of the microvascular changes between CTEPH and IPAH (Moser and Bloor, 1993; Azarian et al., 1997; Yi et al., 2000, Piazza and Goldhaber, 2011), EnMT may contribute to the progression of the microvascular changes in CTEPH.

Recently, we have shown the existence of not only myofibroblast-like cells, but also endothelial-like cells in endarterectomized tissues from patients with CTEPH (Maruoka et al., 2012). Our experiments demonstrated that the endothelial-like cells included a few transitional cells (coexpressing both endothelial- and smooth muscle- cell markers). Moreover, experiments using commercially available HPMVECs and myofibroblast-like cells, which were isolated from the PEA tissues of CTEPH patients, demonstrated that substances associated with myofibroblast-like cells might induce the EnMT (Sakao et al., 2011). Indeed, transitional cells which co-expressed both endothelial- and smooth muscle- cell markers were identified in the PEA tissues of patients with CTEPH (Sakao et al., 2011). In support of our findings, Yao et al. showed the presence of CD34 (an endothelial marker) positive cells co-expressing α-smooth muscle actin (a smooth muscle- cell marker) in endarterectomized tissues from patients with CTEPH (Yao et al., 2009).

As shown in our experiment, Firth et al. demonstrated that a myofibroblast cell phenotype was predominant within endarterectomized tissues from patients with CTEPH, contributing extensively to the vascular lesion/clot (Firth et al., 2010). Moreover, the existence of putative endothelial progenitor cells in endarterectomized tissues of patients with CTEPH has been demonstrated (Yao et al., 2009). Firth et al. have reported the presence of multipotent mesenchymal progenitor cells within the tissues of patients with CTEPH (Firth et al., 2010). These studies suggested that the unique microenviroment created by the stabilized clot may promote these progenitor cells to differentiate into myofibloblast-like cells, and the misguided differentiation of these progenitor cells may enhance intimal remodeling (Yao et al., 2009; Firth et al., 2010). Therefore, myofibloblast-like cells may participate directly in vascular remodeling and they may induce EnMT to lead to EC dysfunction.

Indeed, it may be possible that the cells coexpressing both endothelial- and SM- cell markers in endarterectomized tissues are more likely progenitor cells rather than the cells which are differentiated by EnMT. However, in our *in vitro* experiments, there was no bone marrow-derived cell (defined as born marrow cell markers) in the cultured endothelial-like cells because *ex vivo* conditions may allow these cells to differentiate (Sakao et al., 2011).

EnMT may contribute to the development of vascular remodeling in the patients with CTEPH and interrupting this transition may provide a therapeutic target for CTEPH.

EC dysfunction

The degree and mechanisms of EC dysfunction as a contributor to CTEPH in small muscular arteries distal to nonobstructed pulmonary elastic vessels are unclear (Yi et al., 2000; Dartevelle et al., 2004; Hoeper et al., 2006). However, EC dysfunction may play a crucial role in these areas. Indeed, EC related humoral markers that have been linked to CTEPH include anticardiolipin antibodies, a known risk factor for venous thromboembolism (Torbicki et al., 2008), elevated endothelial factor VIII (Wolf et al., 2000; Bonderman et al., 2003), and monocyte chemoattractant protein 1 (Kimura et al., 2001). Moreover, markers of endothelial trauma or dysfunction, such as endothelins, regularly observed in IPAH, are also found in cases of pulmonary embolism (Sofia et al., 1997). In particular, the endothelin-1 levels in CTEPH closely correlated with the hemodynamic and clinical severity of the disease (Reesink et al., 2006). Endothelin-mediated vascular remodeling and impairment of nitric oxide function may play a crucial role in the development of vascular lesions distal to occluded vessels in CTEPH, as well as in severe PH (Bauer et al., 2002; Reesink et al., 2004). It has been observed that PH is more likely to occur following partial vascular occlusions of pulmonary artery segments than following complete occlusions (Robin et al., 1966), thus suggesting that vasoactive substances produced by the turbulent flow in CTEPH may be involved in EC dysfunction. However, it seems to be difficult to define EC dysfunction in patients with CTEPH.

Several lines of evidence indicate that autophagy has an important role in many different pathological conditions. Moreover, fewer mitochondria, the decreased expression of superoxide dismutase and normoxic decreases in reactive oxygen species have been shown to be the characteristics of mitochondrial abnormalities in PAH (Archer et al., 2008). Our recent findings demonstrated that endothelial-like cells lost their ability to form autophagosomes and had defective mitochondrial structure/function (Sakao et al., 2011), indicating that EC dysfunctions occur in the proximal lesions of patients with CTEPH. Moreover, experiments using commercially available HPMVECs and myofibroblast-like cells demonstrated that factors associated with myofibroblast-like cells might induce HPMVEC dysfunction through the inactivation of autophagy, the disruption of the mitochondrial reticulum, and the improper localization of superoxide dismutase-2 (Sakao et al., 2011). The PCR array data analysis showed that substances associated with myofibroblast-like cells induced the alterations in the endothelial cell biology of HPMVECs (Sakao et al., 2011). Although it is uncertain whether EC dysfunctions actually contribute to microvascular remodeling in patients with CTEPH, the myofibroblast-like cells in the proximal lesions may contribute to EC dysfunction in the vasculature of CTEPH. Indeed, it has been demonstrated that ECs in noninvolved pulmonary vascular beds are different from ECs in regions of organized thromboembolic material in patients with CTEPH (Lang et al., 1994a,b). In patients with CTEPH, primary ECs cultured from pulmonary arteries without thrombus had no abnormalities in the expression of fibrinolytic proteins or responses to thrombin stimulation (Lang et al., 1994a,b). However,

ECs within yellowish-white thrombi, i.e., the highly organized tissues, showed elevated type 1 plasminogen activator inhibitor (PAI-1) mRNA levels (Lang et al., 1994a). Therefore, we have to separate them to consider EC dysfunction.

The correlation between endothelins and vascular remodeling in CTEPH seems to support the possibility that pharmacological therapy using endothelin receptor antagonists are effective treatment for the patients with CTEPH.

In situ thrombosis

ECs not only facilitate the thrombotic process, but also actively inhibit thrombosis and promote fibrinolysis. The production and release of nitric oxide and prostacyclin, two potent inhibitors of platelet aggregation, by ECs are important for the prevention of intravascular thrombosis (Moncada et al., 1991). In addition, the expression of thrombomodulin (TM), a high affinity receptor for thrombin, on the surface of ECs prevents the cleavage of fibrinogen to fibrin. ECs are also a source of tissue plasminogen activator (t-PA), a key activator of plasminogen in the fibrinolytic cascade. On the other hand, ECs also synthesize and release plasminogen activator inhibitor (PAI)-1, an inhibitor of t-PA, highlighting the role of the endothelium in regulating the fine balance between prothrombotic and antithrombotic processes.

Indeed, the plasma concentration of soluble TM in patients with CTEPH was found to be significantly lower than that in the control group, suggesting that a

Table 1. Clinical and pathobiological features of CTEPH.

Hallmarks	Features	Reference	Tissue Culture/ Clinical
Microvascular lesions	Progressive worsening by remodeling in the small distal pulmonary arteries	Azarian et al. (1997)	Clinical
		Moser and Bloor (1993)	Clinical
		Yi et al. (2000)	Clinical
	No hemodynamic improvement and persistent PH despite successful PEA	Condliffe et al. (2008)	Clinical
	microvascular changes in CTEPH similar to those seen in IPAH	Azarian et al. (1997)	Clinical
		Moser and Bloor (1993)	Clinical
		Yi et al. (2000)	Clinical
		Piazza and Goldhaber (2011)	Clinical
	Vasodilative reactivity in the vasculature of CTEPH	Seyfarth et al. (2010)	Clinical
EnMT	Transitional cells in endarterectomized tissues	Sakao et al. (2011)	Tissue Culture
		Yao et al. (2009)	Tissue Culture
	EnMT induced by substances associated with the cells in endarterectomized tissues	Sakao et al. (2011)	Tissue Culture
	The existence of endothelial and mesenchymal progenitor cells in endarterectomized tissues	Yao et al. (2009)	Tissue Culture
		Firth et al. (2010)	Tissue Culture
EC dysfunction	Humoral markers related with EC in CTEPH: Anticardiolipin antibodies Endothelial factor VIII Monocyte chemoattractant protein 1 Endothelins	Torbicki et al. (2008)	Clinical
		Bonderman et al. (2003)	Clinical
		Kimura et al. (2001)	Clinical
		Sofia et al. (1997)	Clinical
		Reesink et al. (2006)	Clinical
	Endothelin-mediated vascular remodeling	Reesink et al. (2006)	Clinical
	The loss of the ability to form autophagosomes	Sakao et al. (2011)	Tissue Culture
	Structure-function defects of mitochondria	Sakao et al. (2011)	Tissue Culture
	ECs with abnormalities in the expression of fibrinolytic proteins or responses to thrombin stimulation	Lang et al. (1994a,b)	Tissue Culture
		Lang et al. (1994a,b)	Tissue Culture
In situ thrombosis	A decreased plasma TM concentration	Sakamaki et al. (2003)	Clinical
	Elevated PAI-1 mRNA levels	Lang et al. (1994)	Tissue Culture
	The decreased expression of Annexin A and plasminogen activator genes in HPMVECs co-cultured with the cells from the PEA tissues	Sakao et al. (2011)	Tissue Culture

CTEPH: Chronic thromboembolic pulmonary hypertension; PH: Pulmonary arterial hypertension; PEA: Pulmonary endarterectomy; IPAH: Idiopathic pulmonary arterial hypertension; EnMT: Endothelial-mesenchymal transition; EC: endothelial cell; TM: Thrombomodulin; PAI-1: type 1 plasminogen activator inhibitor; HPMVECs: Human pulmonary microvascular endothelial cells.

decreased plasma TM concentration might reflect pulmonary vascular EC dysfunction, leading to altered anticoagulant and fibrinolytic function in CTEPH (Sakamaki et al., 2003). ECs within the highly organized tissues in CTEPH exhibited elevated PAI-1 mRNA levels in comparison to patient pulmonary artery specimens that were free of thrombus, suggesting that the prevalence of PAI-1 expression within pulmonary thromboemboli may play a role in the stabilization of vascular thrombi (Lang et al., 1994a). Moreover, there were decreases in the expression of the Annexin A5 and plasminogen activator, urokinase genes in HPMVECs co-cultured with myofibroblast-like cells from the PEA tissues of CTEPH patients (Sakao et al., 2011). Annexin A5 plays an important role in anticoagulant function and is a protein that has a high affinity for negatively-charged phospholipids (Funakoshi et al., 1987; Tait et al., 1988), over which it forms trimers (Voges et al., 1994) that become an annexin A5 shield. The formation of this shield blocks the phospholipids from phospholipid-dependent coagulation enzyme reactions (Andree et al., 1992). Plasminogen activator, urokinase, is a thrombolytic agent. Its primary physiological substrate is plasminogen, which is an inactive zymogen form of the serine protease plasmin. The activation of plasmin triggers a proteolysis cascade that, depending on the physiological environment, participates in thrombolysis or extracellular matrix degradation (Collen and Lijnen, 2005). The decreased expression of Annexin A and plasminogen activator, urokinase, may contribute to the disorder of the anti-coagulation properties in CTEPH patients. However, there is no validation of these data in an *in vivo* experiment.

There are several lines of evidence indicating that EC dysfunction might interfere with the normal balance between the pro-thrombotic and anti-thrombotic mechanisms, resulting in local thrombosis, and may contribute to the pathophysiology of PAH (Eisenberg et al., 1990; Welsh et al., 1996; Wolf et al., 2000). The EC dysfunction in CTEPH may lead to disorder of the anti-coagulation properties in ECs, i.e., may inactivate a vascular fibrinolytic system, and result in the formation of additional clots *in situ*, like PAH, because the histopathological features in the CTEPH vasculature are similar to those seen in IPAH.

Crosstalk between the unresolved clot, EC dysfunction and *in situ* thrombi

Although the first pulmonary embolism is generally accepted as the main initiating event in CTEPH, we hypothesize that the emergence of the microenvironment created by the unresolved clot may result in the local induction of substances that circulate to cause a more widespread predisposition to vascular remodeling affecting the rest of the pulmonary vascular bed, i.e., beyond the site of initial thrombosis. Our recent study suggested that myofibroblast-like cells stimulated by the microenvironment created by the unresolved clot might release substances that promote ECs to transition to other mesenchymal phenotypes and/or induce EC dysfunction, contributing not only to the proximal vasculature, but also to the distal vasculature (Sakao et al., 2011). The precise reasons for the lung-specific action of these substances in CTEPH remain unknown. One explanation may be that the pulmonary vascular

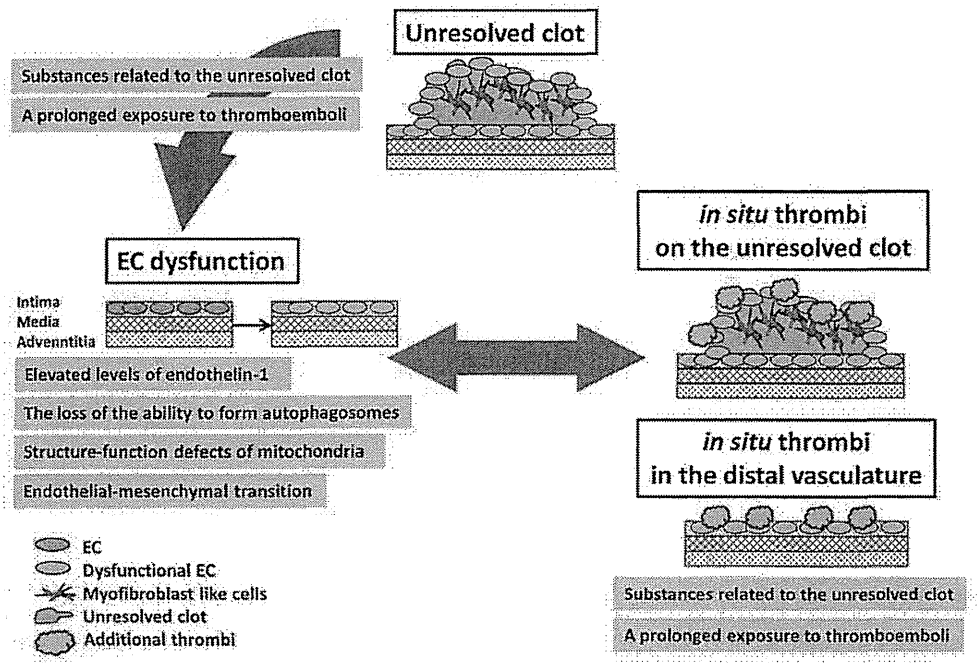

Fig. 1. Crosstalk between EC dysfunction and *in situ* thrombi in CTEPH (a hypothetical mechanism). The cells stimulated by the microenvironment created by an unresolved clot may release substances that induce EC dysfunction. The pulmonary vasculature in patients with CTEPH is subjected to prolonged exposure to thromboemboli. Indeed, thrombin is known to have potent effects on ECs, leading to endothelial barrier dysfunction due to mobilization of Ca^{2+} and rearrangement of the cytoskeleton. An impairment of the EC function in patients with CTEPH may lead to additional thrombi *in situ*, as is seen in patients with PAH, and these may lead to the progression of the proximal clot. A crosstalk between EC dysfunction and *in situ* thrombi may therefore contribute to the vascular lesions of CTEPH. CTEPH: chronic thrombo-embolic pulmonary hypertension, EC: endothelial cells.

beds, i.e., alveolar arteries, are exposed to the highest oxygen tensions in the body, which may induce the different response against substances related by an unresolved clot in comparison to systemic artery ECs. However, this explanation is not sufficient and further explanations are needed.

We fully recognize the limitation of our data interpretation which is based on *in vitro* studies of cultured cells and that this study does not confer any pathological evidence in CTEPH. Indeed, extensive small vessel disease may be a complication of a minority of CTEPH cases. Therefore, besides substances related to the microenviroment created by the stabilized clot, a second factor may be required to induce EC dysfunction which results in extensive disease. However, it remains unknown what is the second factor that is responsible for whether extensive small vessel disease occurs in a given patient.

In the pathogenesis of CTEPH, pulmonary microvascular lesions develop in the distal areas of unoccluded as well as occluded pulmonary arteries (Moser and Bloor, 1993; Azarian et al., 1997; Yi et al., 2000). The development of microvascular lesions distal to totally obstructed pulmonary arteries may be promoted by substances related to the microenvironment created by the unresolved clot. The development of the lesions distal to nonobstructed pulmonary arteries may be promoted not only by substances, but also by increased shear stress caused by hypoxic pulmonary vasoconstriction, because shear stress has been shown to inhibit apoptosis of ECs (Pi et al., 2004) and to stimulate EC growth (Ameshima et al., 2003; Sakao et al., 2005), contributing to vascular remodeling. However, unless the occlusion is enormous, it seems unlikely that vessel occlusion alone increases shear stress in unoccluded arteries because of the large reservoir capacity of the normal pulmonary vasculature. A more likely explanation for the lesions distal to nonobstructed pulmonary arteries may be that the pulmonary arteriopathy could be the initial pathology of the lesions in the patients with IPAH (Peacock et al., 2006). In any case, a persistent clot in the peripheral pulmonary arteries despite successful PEA may continue to create the microenvironment that induces microvascular changes. This may be the reason why there are patients who do not respond to PEA.

In the proximal lesions in patients with CTEPH, the pulmonary vasculature is subjected to a prolonged exposure to thromboemboli, i.e., components in the final common pathway of the coagulation cascade. Indeed, thrombin, a serine protease that catalyzes the conversion of fibrinogen to fibrin, is known to have potent effects on ECs, leading to endothelial barrier dysfunction due to the mobilization of Ca^{2+} and rearrangement of the cytoskeleton (Ellis et al., 1999). Moreover, chronic exposure to fibrinogen, fibrin, and thrombin caused changes in the cytosolic Ca^{2+} in pulmonary artery ECs, suggesting that such changes might contribute to EC dysfunction, thus leading to vascular changes in patients with CTEPH (Firth et al., 2009).

Based on these observations, it has been suggested that many kinds of insults to ECs of the pulmonary arteries may initiate a sequence of events which leads to the EC dysfunctions in CTEPH. Numerous factors such as hypoxia, endogenous vasoconstrictors, and inflammatory cytokines could help to sustain this process (Egermayer et al., 1999). An impairment of the EC function in patients with CTEPH may lead to additional thrombi *in situ* similar to that observed in patients with PAH, and these may also lead to the progression of the proximal clot.

It has been suggested that the core of the pathological process in CTEPH is not only related to thrombus formation, but it is also linked to disturbed thrombus resolution (Morris et al., 2006, 2007; Suntharalingam et al., 2008). An altered coagulation process may account for the pathological features of CTEPH (Wolf et al., 2000). Recently, the fibrinogen A Thr312Ala polymorphism was shown to correspond to significant differences in the genotype and allele frequencies between CTEPH and control subjects. The presence of these polymorphisms may confer resistance to fibrinolysis that subsequently contributes to the development of thrombus organization (Suntharalingam et al., 2008). The other mechanism may be the development of more fibrinolysis-resistant fibrin clots from patients with CTEPH, when compared with the fibrin clots from healthy control subjects (Morris et al., 2006). An abnormally elevated amount of disialylated fibrinogen γ-chain can render a clot resistant to plasmin, which could lead to the subsequent development of CTEPH (Morris et al., 2007). However, these explanations are not sufficient because there are many patients without known coagulation problems who have these factors, and because numerous genetic variants of human fibrinogen have been implicated in thrombotic diseases (Matsuda and Sugo, 2002). Therefore, the resistance could be ascribed to not only fibrinogen genetic polymorphisms, but also variations in the post-translational modifications.

Conclusion

Besides the altered coagulation process, a crosstalk between EC dysfunction and *in situ* thrombi may contribute to the vascular lesions of CTEPH (Fig. 1) (Table 1). Moreover, this may explain why pulmonary thromboemboli in CTEPH patients are stable. Indeed, pulmonary thromboendarterectomy may be the best way to break this crosstalk. Recently, we demonstrated that poor subpleural perfusion on pulmonary angiography might be related to distal vascular remodeling and an inadequate surgical outcome of CTEPH (Tanabe et al., 2012). No satisfactory hemodynamic improvement and persistent PH despite successful PEA in the patients with CTEPH (Condliffe et al., 2008) suggests the existence of distal vascular remodeling. Although it remains uncertain whether vascular remodeling is actually related

to the crosstalk between EC dysfunction and *in situ* thrombi, the care for these patients should be directed toward pharmacologically reducing pulmonary vascular resistance with specific vasodilative compounds as used for PAH therapy. The next step in the future is to find out new ways to define EC dysfunction and vascular remodeling in CTEPH objectively.

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Prevalence and clinical features of lymphedema in patients with lymphangioleiomyomatosis

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Lymphoscintigraphy

Summary
Background: Lymphangioleiomyomatosis (LAM) is a rare cystic lung disease predominantly affecting young women. Some of these patients develop lymphedema of the lower extremities and buttocks; however, neither the exact frequency of LAM-associated lymphedema nor the clinical features of such patients is well delineated.
Objectives: To document the frequency, features, and treatment of LAM-associated lymphedema.
Methods: We reviewed all medical records of patients listed in the Juntendo University LAM registry for the 30 years preceding August 2010.
Results: Of 228 patients registered with a diagnosis of LAM, eight (3.5%) had LAM-associated lymphedema of the lower extremities. All were females with sporadic LAM, and their mean age when diagnosed was 32.5 years (range 23–44). Lymphedema of the lower extremities was the chief or a

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prominent presenting feature in five of these LAM patients. CT scans showed that all eight patients had enlarged lymph nodes (lymphangioleiomyomas) in the retroperitoneum and/or pelvic cavity. Yet, cystic destruction of the lungs was mild in four patients, moderate in two and severe only in two. Seven of these patients were treated by administering a fat-restricted diet and complex decongestive physiotherapy, and four received a gonadotropin-releasing hormone analog. With this combined protocol, all eight patients benefitted from complete relief or good control of the lymphedema.

Conclusions: Lymphedema is a rare complication of LAM and may be associated with axial lymphatic involvement or dysfunction rather than severe cystic lung destruction. The combined multimodal treatments used here effectively resolved or controlled LAM-associated lymphedema.

Introduction

The neoplastic disease lymphangioleiomyomatosis (LAM) predominantly affects the lungs of young women but also occurs along the axial lymphatic systems, including lymph nodes in the mediastinum, retroperitoneum, pelvic cavity, and thoracic ducts. This rare disease is characterized by the proliferation of LAM cells (smooth muscle-like cells) and LAM-associated lymphangiogenesis. LAM cells are considered to be transformed by abnormalities of either the *TSC1* or *TSC2* tumor suppressor gene.[1,2]

The clinical manifestations of LAM include exertional dyspnea, spontaneous pneumothorax, hemosputum, chylous pleural effusion, and symptoms associated with extrapulmonary involvement.[3] The representative extrapulmonary problems are chylous ascites and lymphangioleiomyomas (of axial lymph nodes and lymphatics) in the retroperitoneum and pelvic cavity. Lymphangioleiomyomas usually do not exhibit symptoms. However, some LAM patients may feel unexplained pain or distension and also lymphedema of the lower extremities and buttocks.

Lymphedema is the result of protein-rich interstitial volume overload, secondary to lymph drainage failure in the face of normal capillary filtration.[4] This state occurs when there is an inherent defect within the lymph-carrying conduits, termed "primary lymphedema," or damage arises, termed "secondary lymphedema" (e.g., pressure from tumors, scar tissue after radiation, surgical removal of lymph nodes, etc.). Because lymphedema is often difficult to cure, the result may be such psychological sequelae as frustration, distress, depression and anxiety.[5,6] For many of these patients, the quality of life becomes impaired.

Since the exact frequency of LAM-associated lymphedema and clinical features of LAM patients whose condition is complicated by lymphedema are not well delineated, we retrospectively reviewed our LAM registry. The purpose was to understand and alleviate the troubling outcome of this disease.

Methods

Identification of patients with LAM-associated lymphedema

As of August 2010, we retrospectively reviewed medical records of patients with LAM who visited the Department of Respiratory Medicine, Juntendo University Hospital, and were recorded in the Juntendo LAM registry since 1980. We found LAM-associated lymphedema of the lower extremities in eight (3.5%) of 228 LAM patients. We then analyzed followup notations from their medical records: that is, age at the diagnosis of LAM, symptoms, radiological findings on scans from the chest to pelvic cavity based on either computed tomography (CT) or magnetic resonance imaging (MRI), and treatment for LAM and LAM-associated lymphedema. Lymphedema was diagnosed from physical examination, imaging (lymphoscintigraphy, MRI, CT and/or ultrasonography) and exclusion of other diseases that cause edema in the lower extremities.

Grading of cystic lung destruction

The severity of cystic lung destruction due to LAM was assessed visually according to the modified Goddard scoring system.[7] Six images were selected (bilateral lung field in the upper, middle, and lower axial lung slices) and analyzed. Each image was classified as normal (score 0), $\leq$5% affected (score 0.5), $\leq$25% affected (score 1), $\leq$50% affected (score 2), $\leq$75% affected (score 3), >75% affected (score 4), giving a minimum score of 0 and maximum of 4. An average score of all images was considered as a representative value of the severity of cystic destruction (mild, score <1; moderate, 1–2.5; and severe, >2.5).

Classification of lymphedema

The severity of lymphedema was determined from classifications of the International Society of Lymphology[8] and is summarized briefly as follows. Stage 0 refers to a latent or sub-clinical condition in which swelling is not evident despite impaired lymph transport. Stage I represents an early period of the condition when the accumulated fluid is relatively high in protein content but subsides with limb elevation. Pitting may occur in stage I lymphedema. Stage II signifies that limb elevation alone rarely reduces tissue swelling, and pitting is manifested routinely. Stage III encompasses lymphostatic elephantiasis without pitting but includes trophic skin changes.[8]

Complex decongestive physiotherapy (CDP)

We performed complex decongestive physiotherapy (CDP), which is a two-phase noninvasive therapeutic regimen,

according to the study reported by Ko et al.[9] Briefly, the first phase treatment consists of manual lymph massage (MLM), compression therapy (multilayered inelastic compression bandaging/elastic stocking), remedial exercises, and skin care. The phase two treatment focuses on continuous self-care at home by means of daytime elastic stocking compression, MLM, and continued exercises. Since some LAM patients had advanced lung involvement or were complicated with chylous pleural effusion, chylous ascites, or both, some components of the first phase treatment were needed to be modified (the intensity and duration of MLM, type and material of bandaging, degree of compression applied by elastic garments, etc.) or customized (exclusion of compression therapy and remedial exercise), depending on the patient's clinical condition.

Results

Prevalence and clinical features of LAM-associated lymphedema

We reviewed medical records of 228 patients with LAM (203 with sporadic LAM and 25 with TSC-LAM) who visited our hospital from 1980 to August 2010. Among them, eight female patients (3.5%) were identified as having LAM-associated with lymphedema in the lower extremities and buttocks (Table 1). All 8 LAM patients were sporadic LAM. In contrast, none of TSC-LAM in our cohort complicated LAM-associated lymphedema in the lower extremities and buttock. One TSC-LAM patient with lymphedema in the lower extremities was excluded since it was due to the surgical resection of intrapelvic tumor (uterine anigosarcoma).[10] The patients' mean age at the diagnosis of LAM was 32.5 years (range 23–42), and their mean age at the onset of lymphedema was 33.4 years (range 23–42).

Notably, lymphedema of the lower extremities was an important determinant or confirming factor in the diagnosis of LAM for five patients; the remaining three patients had lymphedema transiently or occasionally during their disease course. Usually, the lymphedema occurred in the lower limbs (seven of eight patients) with the left leg more frequently involved than the right, and two patients had these swellings in bilateral lower extremities. Patient #6 (JUL216, 42-years-old) had lymphedema of her left thigh with a skin color change that induced her to seek a medical evaluation and eventually led to the diagnosis of LAM (Fig. 1). Two patients {patient #4 (JUL181) and patient #5 (JUL213)} had lymphedema in the buttocks as well as lower extremities. Patient #7 (JUL221) had transient lymphedema around the left lower abdominal area only when she wore tight underwear (Fig. 2B). The severity of lymphedema was

Table 1 Clinical features of study population with LAM-associated lymphedema.

	Patient #1	Patient #2	Patient #3	Patient #4	Patient #5	Patient #6	Patient #7	Patient #8
Registry number	JUL112	JUL127	JUL130	JUL181	JUL213	JUL216	JUL221	JUL129
Age at the diagnosis of LAM	28	27	40	35	23	42	33	32
Age at the onset of lymphedema	28	27	44	37	23	42	34	32
History of PTX	−	−	+	−	+	−	−	+
Presenting feature	Lymphedema	Lymphedema	PTX	DOE	Lymphedema	Lymphedema	Medical checkup	Lymphedema
Site of lymphedema	Bilateral LE	Right LE	Left LE	Right LE Buttock	Bilateral LE Buttock	Left LE	Lower abdomen	Left LE
Stage of lymphedema								
before treatment	2	2	2	2	2	2	1	2
after treatment	0	0	0	0	1	1	0	0
Serum VEGF-D (pg/ml)	1357	6080	10,900	16,800	10,869	7398	1904	3010
Severity of cystic destruction on chest CT	Mild	Severe	Moderate	Severe	Moderate	Mild	Mild	Mild
Lymphangioleiomyomas	R	R	R + Pv	R + Pv	Inguinal	R + Pv	Pv	R + Pv
Chylous pleural effusion	−	+	−	−	+	−	−	−
Chylous ascites	−	+	+	+	+	−	−	−
Angiomyolipoma	−	−	Left kidney	−	−	−	−	−
Treatment	FRD, CDP	FRD, CDP, GnRH, HOT	FRD, CDP, GnRH	FRD, CDP, GnRH, HOT	FRD, CDP, GnRH, HOT	FRD, CDP	Instruction only	FRD, CDP

Abbreviations: CDP, complex decongestive physiotherapy; DOE, dyspnea on exertion; FRD, fat-restricted diet; GnRH, gonadotropin-releasing hormone analog; HOT, home oxygen therapy; LE, lower extremity; PTX, pneumothorax; R, lymphangioleiomyomas in the upper abdominal region of retroperitoneum; and Pv, lymphangioleiomyomas in the pelvic cavity.

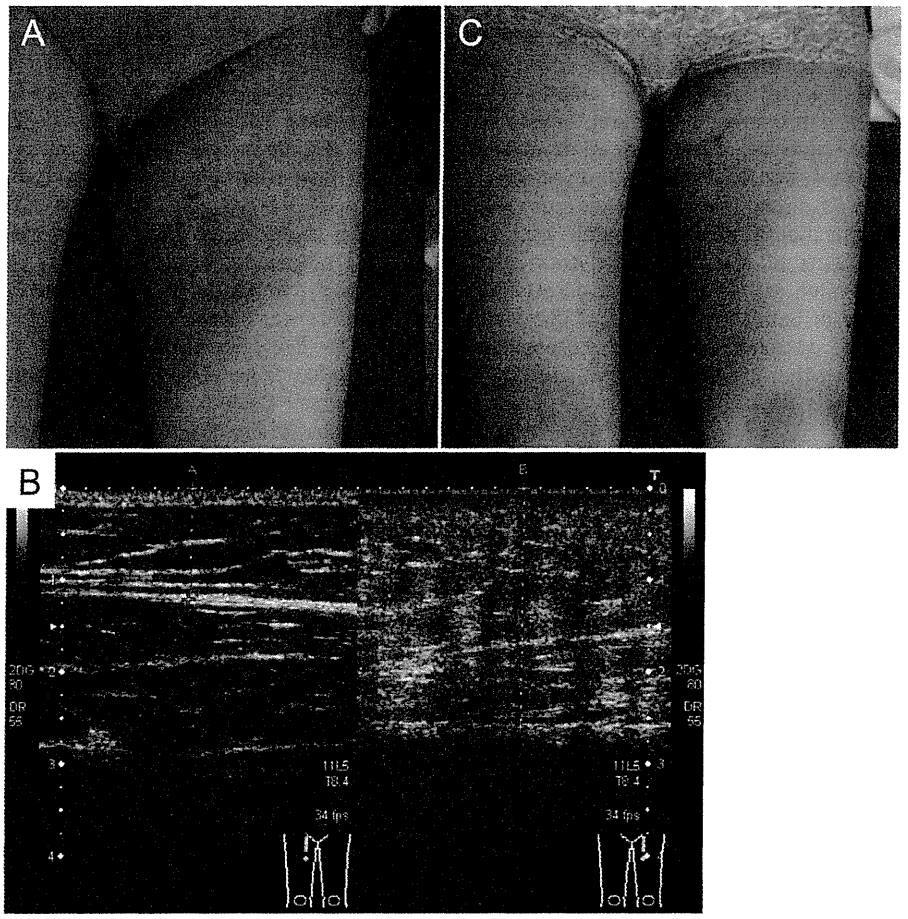

Figure 1 A representative portrait of LAM-associated lymphedema (patient #6, JUL216). Here, the left thigh was swollen, and inner side of its proximal portion showed rubor (A). Ultrasonography of both thighs revealed increased intensity and widening of the subcutaneous layer only in the left thigh (B). In panel B, the scan on the left illustrates the right thigh, and that on the right pictures the left thigh, supporting the diagnosis of lymphedema (stage 2). After CDP, lymphedema of left thigh decreased from stage 2 to 1 (C).

classified as stage 2 in seven of these eight patients; only patient #7 was at stage 1. Lymphoscintigraphy was performed on three patients and enabled the identification of a blockade that impeded normal axial lymphatic flow as shown in the representative example appears of Fig. 3.

Clinical features of patients with LAM and associated lymphedema

The pulmonary and extrapulmonary findings of LAM as well as treatments administered for LAM and LAM-associated

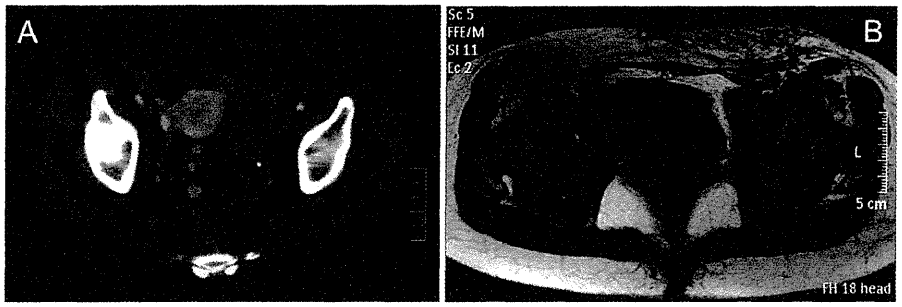

Figure 2 Representative images of lymphangioleiomyomas in the pelvic cavity and lymphedema of the lower abdominal skin (patient #7, JUL 221). Computed tomography of the pelvic cavity after intravenous injection of contrast material revealed cystic lymphangioleiomyomas along the left internal iliac vessels (A). T1-weighted MRI axial images without contrast material demonstrated thickening and fluid accumulated in the subcutaneous tissue of the left lower abdominal skin (B).

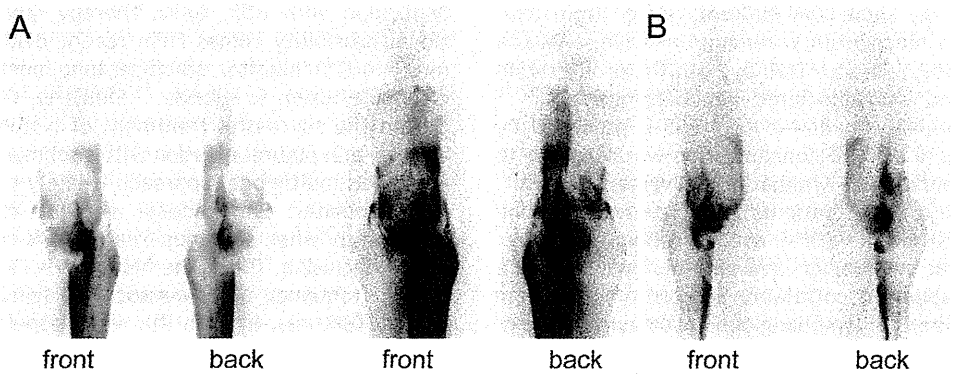

Figure 3 Lymphoscintigraphy of the lower extremities (patient #5, JUL213). This lymphoscintigram scanned 60 min after the subcutaneous injection of ^{99m}Tc-labeled human serum albumin (^{99m}Tc-HAS) at instep (A, left and B, right) revealed a marked dermal backflow of ^{99m}Tc-HAS due to a blockade of the normal axial lymphatic flow. A: left lower extremity with two differentially gained-images. B: right lower extremity.

lymphedema are summarized in Table 1. With respect to findings, the extent of cystic destruction of the lungs was mild in four patients, moderate in two and severe in two. Two patients {patient #2 (JUL127) and patient #5 (JUL213)} showed ground glass attenuation of the lungs suggesting lymphedema. However, there was no correlation between the severity of cystic destruction of the lung and LAM-associated lymphedema. All eight patients had lymphangioleiomyomas (enlargements of lymph nodes and/or lymphatics): seven patients had this condition in the retroperitoneum from an upper abdominal area to the pelvic cavity (Fig. 2A) but only patient #5 was similarly affected in the right inguinal area. Chylous pleural effusions were present in two patients and chylous ascites in four of them. Moreover, two patients suffered chylous pleural effusion and also ascites. An angiomyolipoma was evident in only one patient {patient #3 (JUL130)}. Measurement of the serum vascular endothelial growth factor (VEGF-D) showed a concentration of more than 800 pg/ml, indicating a possible diagnosis of LAM (Table 1).

Treatment for these patients varied over the long term of this study. Seven patients were treated with a fat-restricted diet (FRD) and complex decongestive physiotherapy (CDP). Four received monthly subcutaneous injections of gonadotropin-releasing hormone (GnRH) analog. The CDP, which was administered to seven patients, provided complete relief or substantial control of their lymphedema (Fig. 1B). For patient #7 (JUL221), no specific treatment was required, since wearing less restrictive underwear was enough to resolve her lymphedema.

Discussion

For the present study, we established the frequency of LAM-associated lymphedema over a 30-year-period in one hospital and analyzed the clinical features of patients whose LAM was complicated with lymphedema. Lymphedema was noted in 3.5% (8 of 228) of these LAM patients and was the main or at least an important presenting symptom of LAM in five of the eight patients. Therefore, the frequency of lymphedema as a presenting feature of LAM in this group was 2.2% (5/228). In none of these patients did the severity of lymphedema exceed the classification of stage 2 or less. Prior to our study, only three case reports about LAM-associated lymphedema of the lower extremities appeared in the literature,[11–13] and its exact incidence remained unknown.

The diagnosis of LAM as an underlying cause of lymphedema is often challenging. Generally, secondary lymphedema is caused by axillary, pelvic or inguinal lymph node dissection and/or radiation for the treatment of malignancy or infections, those diseases or conditions eventually resulting in the obstruction of lymphatics. Accordingly, the diagnosis of secondary lymphedema would not be difficult if symptoms or a past history indicated an impact on lymphatic drainage. Similarly, physical findings such as non-pitting edema and Stemmer sign would serve as clues. Furthermore, secondary lymphedema typically involves a single limb, whereas more widespread involvement may be seen in primary hereditary lymphedema.[14] In our study, LAM was at an early stage (mild degree), most often with unremarkable physical findings. In 25% of these patients, lymphedema developed in both lower extremities. Therefore, it is worth emphasizing that here and elsewhere, ultrasonography and MRI have been useful for diagnosing the lymphedema and that lymphoscintigraphy helped to delineate the functional and physiological derangement of lymphatic flow in the affected areas (Figs. 1–3).[14–16] However, even if we can establish the diagnosis of lymphedema, LAM would not be considered as an underlying disease unless characteristic LAM-related symptoms and findings such as exertional dypnea, pneumothorax, chyle leak, etc., are present. Instead, lymphomas or soft tissue neoplasms would most likely be suspected, since enlarged lymph nodes (lymphangioleiomyomas) mimicking abdominal tumors can be demonstrated in the retroperitoneum, pelvic cavity, or inguinal area. Indeed, all eight LAM patients with lymphedema in their lower extremities or lower abdomen had lymphangioleiomyomas that were then extirpated completely in the three patients (patients #1, #2, and #8) whose clinical diagnoses suggested soft tissue neoplasm or lymphoma.