Current Topics of Physiology and Pharmacology in the Lymphatic System

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Abstract

We have reviewed physiological significance of rhythmical spontaneous contractions of collecting lymph vessels, which play a pivotal role in lymph transport and seem to control lymph formation through changing the pacemaker sites of the rhythmic contractions and contractile patterns of the lymphangions. A characteristic feature that the rhythmic pump activity works in vivo physiologically under the specific environment of lower oxygen tension in lymph (25-40 mmHg) has been evaluated. With the characteristic feature, generation of endogenous nitric oxide (NO) from lymphatic endothelial cells and/or activation of ATP-sensitive potassium channels (K_{ATP}) are reviewed to play crucial roles in the regulation of lymph transport at physiological or pathophysiological conditions. Chemical substances released from malignant tumor cells and tumor-derived parathyroid hormone-related peptide (PTHr-P) are also shown to cause a significant reduction of lymphatic pump activity through generation of endogenous NO and activation of K_{ATP} channels. Finally, we have discussed physiological significance and roles of the lower oxygen tension in lymph, generation of endogenous NO, and activation of K_{ATP} in lymph formation, lymph transport, and the functions of lymph nodes.

KEY WORDS; Lymphatic system, spontaneous contraction, lymphatic endothelial cell, oxygen tension, nitric oxide, prostaglandins, reactive oxygen radicals, K_{ATP} channel, tumor-derived chemical substance, ATP, adenosine, PTHr-P

ABBREVIATIONS; ACh, acetylcholine; AM, acetoxymethyl ester; ATP, adenosine tri-phosphate; bFGF, basic fibroblast growth factor; $[Ca^{2+}]$, intercellular concentration of calcium ions; COX, cyclo-oxygenase; DiI-Ac-LDL, 1,1-diocadecyl 1-3,3,3',3'-tetramethylindo-carbocyanine perchlorate-labeled acetylated low-density lipoprotein; Dmax, maximum diameter; Dmin, minimum diameter; DMPX, 3,7-dimethyl-1-proparglyxanthine; DPCPX, 8-cyclopentyl-1, 3-diprophylxanthine; EDD, end-diastolic diameter; EF, ejection fraction; EGM-2, endothelial growth medium-2; ESD, end-systolic diameter; ET, endothelin; F, frequency of lymph pump activity; FITC, fluorescein 5'-isothiocyanate; GTP, guanosine tri-phosphate; 5-HT, 5-hydroxytryptamine; K_{ATP} channel, ATP-sensitive potassium channel; LEC, lymphatic endothelial cell; LLC, Lewis lung carcinoma; L-NAME, N^{w}-nitro-L-arginine; L-NMMA, N^{G}-monomethyl-L-arginine; LYVE-1, lymphatic vessel endothelial hyaluronan receptor; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, 1*H*-[1,2,4,] Oxadiazolo[4,3-a]quinoxalin-1-one; PDGF, platelet-derived growth factor; PFI, pump flow index; PGs, prostaglandins; PO_2, partial pressure of oxygen; Prox-1, prospero-related homeobox 1; PTHr-P, parathyroid hormone-related peptide; ROS, reactive oxygen radicals; SLN, sentinel lymph node; SNP, sodium nitroprusside; STD, spontaneous transient depolarization; SV, stroke volume; TX, thromboxane; VEGF, vascular endothelium growth factor; VEGFR, vascular endothelium growth factor receptor

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1.INTRODUCTION

The functions of lymph vessels and lymph nodes as a tissue drainage system and an immuno-surveillance have been recognized for well over a century. In general, in all parts of the body the large lymph vessels act as means for return to the circulation of fluid that leaks out of blood capillaries into the tissues, especially plasma protein, that leak out of the venules and can not be reabsorbed directly back into the circulation. The terminal vessels of the lymphatic system are very minute lymphatic capillaries that lack, in contrast to blood capillaries, fenestrations, a continuous basal membrane and pericytes; instead, these vessels are lined with a continuous, single layer of overlapping endothelial cells that form loose intercellular junctions. These characteristics make the lymphatic capillaries highly permeable to large macromolecules; pathogens and migrating cells. Lymph returns to the venous circulation through the thoracic duct and the collecting lymph vessels, which contain a muscular layer and adventitial layer.

Located along the lymph trunks are lymph nodes through which the lymph flows. These nodes act as filters to remove particular matter from the lymph before it flows into the circulation. The reticuloendothelial cells of the lymph nodes phagocytize most of the particulate matter, and they form immune bodies against such invading agents as bacteria, toxins, and so forth [Yoffey and Courtice, 1970; Guyton et al., 1975].

On the other hand, interest in basic lymphatic research has radically increased in the past few years. It may be, in part, related to the identification of lymph vessel with specific markers such as lymphatic vessel endothelial hyaluronan receptor 1(LYVE-1), prospero-related homeobox 1 (Prox-1), podoplanin, and vascular endothelial growth factor receptor 3 (VEGFR3) and growth factors such as VEGF C and D. The hot debate about tumor-mediated lymphangiogenesis also contributes to the development of basic lymphatic research [Oliver, 2004]. Malignant tumors can directly activate lymphangiogenesis and lymphatic metastasis [Karpanen et al., 2001; Mandriota et al., 2001; Skobe et al., 2001; Stacker et al., 2001; Wigle et al., 2002].

Metastasis of most cancers mainly occurs through the lymphatic system, and the extent of lymph node involvement is a useful prognostic indicator. Thus the status of the regional lymph node is known to remain the most powerful predictor of survival in women with invasive breast cancer, and this status is used to make treatment decisions [Morrow 1996; Baxter et al., 1996]. Therefore Morton et al. (1992) for melanoma and Giuliano et al. (1994) for breast cancer proposed originally

a procedure, in which lymphatic drainage from primary tumors can be mapped to the regional lymph nodes. With the mapping, they established a concept of sentinel lymph node(s) (SLN) that is the first node(s) draining the primary tumor in the lymphatic network. This SLN is the presumptive initial site of the lymphatic metastasis of carcinoma cells and the histological characteristics of the SLN reflect those obtained with the rest of lymph nodes in the lymphatic networks.

Despite taking accounts with the clinical importance of tumor-mediated lymphangiogenesis, the concept of SLN, and the belief that metastatic tumor cells originated from epithelium are disseminated mainly through the lymphatic vasculature, the most basic aspects of lymph formation and transport in the lymphatic system remain to be clarified. Considerable progress has been made at the ultrastructural, microcirculatory, and pharmacological level [Ohhashi 1987, 1993, 1994; Roddie 1990; Schmid-Schönbein 1990; Aukland and Reed 1993; Swartz 2001]. However, we have not arrived at an unity of picture of regulating mechanisms of lymph formation and lymph transport in physiological and pathophysiological conditions, especially lymphangiogenesis, lymphatic metastasis of carcinoma cells, and development of the SLN. In this review, we emphasize current physiological and pharmacological topics of the regulating mechanisms of active lymph transport with intrinsic rhythmic pump mechanisms with special reference to lower oxygen tension in lymph, generation of endogenous nitric oxide (NO) and reactive oxygen radicals (ROR), and activation of ATP-sensitive potassium (K_{ATP}) channels. In future perspectives, we also discuss physiological significance and crucial roles of slow movement of plasma proteins through the lymphatic system, almost of which are mainly leaked out from venules. We have proposed new hypothesis that the movement of plasma proteins through the lymphatic system plays a key role in the regulation of innate immunity through controlling the release of lymphocytes including natural killer (NK) cells from regional lymph nodes.

2. Intrinsic lymphatic pump mechanisms

An important aspect of the lymphatic pump derives from newly discovered structural features of lymphatic capillaries [Leak and Burke 1968a & b]. The two most important items of these features are as follows. (1) The endothelial cells forming the lymphatic capillary are not bound tightly to each other but, instead, simple overlap. (2) The endothelial cells are hold tightly to surrounding tissues by anchoring filaments, which attach to all parts of the endothelial cells except to the internal flap of each cell where it overlaps its adjacent cell. These two structural

features allow fluid to enter the lymphatic capillary whenever the pressure outside the capillary is greater than the pressure inside. The fluid merely pushes the endothelial flap toward the interior of the capillary and wends its way into the capillary lumen. Then, whenever the pressure inside the capillary becomes greater than that outside, the endothelial flap closes over the space between the endothelial cell, and the fluid can not escape. Thus, a lymphatic pump operates at the very tips of the lymphatic capillaries, because any compression and relaxation of the tissues, or of the lymphatic capillaries themselves will create alternating pressure differences across the capillary membrane. Fluid moves out of the lymphatic capillary and up the collecting lymph vessels during the compression cycle. Then, during the relaxation cycle, it moves into the lymphatic capillary from the surrounding tissue spaces. Therefore, our conception of the function of the lymphatic system has changed from that of a passive system to one with not only an active role but, indeed, a strongly active role. In fact, the findings go so far as to demonstrate that the lymphatic capillary endothelial cells themselves have a contractile nature (See Figure 7) and that their cytoplasm actually contains actomyosin [Guyton et al., 1975].

Currently it is also very clear that the rhythmical activity of lymph vessels, combined with the presence of valves inside these vessels, can create pumping pressures as great as 20 to 30 mmHg [Hall et al., 1965]. Therefore, the lymphatic system is, in effect, a sump pump for the tissues, always attempting to propel excess free fluid away from the tissue spaces. In some animals, including man, sheep, cattle, rat, and mouse, the larger collecting lymph vessels have shown to undergo spontaneous rhythmic contractions [Kinmonth and Taylor, 1956; Hall et al., 1965; Mawhinney and Roddie, 1973; Ohhashi et al., 1980; Benoit et al., 1989; Ono et al., 2000]. The frequency of contractions seems to be determined mainly by the amount of fluid in the lymph vessel. When a segment of vessel immediately below a valve becomes distended, it contract, and the fluid is pushed forward beyond the valve. The excess filling on the upstream side of this value causes the next segment of the lymphatic vessel to contract, thus propelling the fluid forward still another segment. In other words, each segment of lymphatic vessel operates as a separate individual pump and is responsive to the amount of lymph that fills its chamber. Thus the lymphatic pump activity is defined as active propulsion mechanisms of lymph mediated by rhythmical spontaneous contractions of lymphatic smooth muscles.

2.1 Mechanical and electrical characteristics of spontaneous contractions

Studies in isolated lymphatic preparations have been of great importance for excluding passive external forces, and for characterizing the contractile process. Mislin (1961) started firstly such in vitro experiments, and introduced the term “lymphangion” for an intervalve segment as the contractile functional unit. Waldeck (1965) extended these observations by recording active and passive pressure-volume curves in isolated three- to five-valve segments of hepatic and mesenteric lymph vessels from rats. He found that the contractile strength increased to a maximum at increasing transmural pressure and then fell off. The contractions of the fine lymph vessels (volume, 0.5 μl) were able to raise the luminal pressure by only 1-2 mmHg. A similar volume-pressure diagram was obtained by us (1980) on an isolated lymphangion from bovine mesentery (volume, 300 μl) giving a maximal pulse pressure of 20 mmHg, as shown in Figure 1. Even higher “systolic pressure”, 60 –120 mmHg, have been shown to be generated in obstructed popliteal lymph vessels in sheep [Hall et al., 1965] and in human legs [Olszewski and Engeset, 1980].

An excellent characterization of the pumping properties of intact mesenteric collecting lymphangions in rats was obtained by Benoit et al. (1989) combining continuous diameter and pressure measurements and analyzing the data in terms used to describe cardiac function. Under control conditions, the contraction frequency was 6.4 min^{-1}, the shortening velocity was very high compared with that of other types of smooth muscles, and the ejection fraction was 67 %. Increasing lymph formation by intravenous saline infusion increased end-diastolic volume, contraction frequency, and stroke volume; while criteria for inotropic effects, such as ejection fraction and the rate of systolic pressure increase, showed less consistent results.

Tetrodotoxin fails to abolish the spontaneous contractions [Azuma et al., 1977], suggesting a myogenic pacemaker that functions well without nerve stimuli. A dominant pacemaker site seems to be located in each lymphangion immediately downstream to each valve [Ohhashi et al., 1980], probably in the circular muscle layer. Within a given lymphangion the contraction spreads at a velocity of 4-5 mm/s [Ohhashi et al., 1980], suggesting a cell-to-cell propagation as in cardiac or visceral smooth muscle. While single contractions seems to be the rule during free flow, lymphatic obstruction and increasing pressure in human leg lymph vessels induce bursts of four to seven rapidly repeated contractions interspersed with silent intervals [Olszewski and Engeset, 1980]. An activation threshold of 5 – 10 mmHg

was found in these vessels. The importance of the rate of pressure change was pointed out by us [Ohhashi et al., 1980], showing that both a rise and a rapid fall in transmural pressure may initiate contraction in isolated bovine mesenteric lymphangions.

We studied the electrical activity corresponding to the spontaneous contractions of lymphatic smooth muscles by use of sucrose gap [Azuma et al., 1977] and intracellular microelectrode techniques [Ohhashi et al., 1978; 1982]. The mean resting membrane potential of the lymphatic smooth muscle cells is about – 50 mV. The resting membrane potential sometimes shows rhythmic fluctuations or slow waves that are resemble those in visceral smooth muscles [Speden, 1964]. The minimum depolarization necessary for inducing the spontaneous contraction is about 6 mV in the lymphatic smooth muscle cells [Ohhashi and Azuma, 1982]. In potassium-free solution, the resting membrane potential is depolarized by about 9 mV, and then the lymphatic smooth muscles appear a sustained contraction. Ouabain at 10^{-5} M also causes a depolarization of the membrane potential with a tetanic contraction in isolated bovine mesenteric lymph vessels. The findings suggest that changes of membrane potential seems to play a significant role in the activation of contractile proteins in the lymphatic smooth muscles and that there exists an electrogenic sodium pump on plasma membrane of the smooth muscle cells. The depolarization and tension development in the potassium-free solution may be due to a decreased activity of the electrogenic sodium pump on the lymphatic smooth muscle cells.

The spontaneous contraction is accompanied by the production of action potentials in the lymphatic smooth muscle cells. The action potential could be classified into two patterns, i.e., (1) short trains consisting of several spikes, and (2) single spikes. Occasionally the action potentials are superimposed upon the rising phase of the slow fluctuation [Ohhashi et al., 1978]. Slow inward calcium current may play a major role in producing the spike discharge in the lymphatic smooth muscles [Azuma et al., 1977]. Individual contractions are the result of a single action potential.

2.2 Peristalsis- and pendular-type spontaneous contractions

As earlier described by Baez (1960), although a contraction usually appears to the observer as an instantaneous total contraction of the vessel wall, its progressive peristaltic character was clearly demonstrated by high-speed cinemicrophotography, as confirmed by us (1980) and Benoit et al. (1989). However, according to Mislin

and Rathenow (1962), contraction waves may also spread in upstream direction over several segments, without being elicited by a rise in local transmural pressure.

Other investigators have described propagated peristaltic waves and suggested that contraction and emptying of one segment would rise the pressure in the next lymphangion and thereby trigger its pacemaker [Hall et al., 1965; Waldeck, 1965]. Mathematical models based on this concept led to conclude that the confluence of vessels, finally converging on the thoracic duct, may cause an irregular contraction rhythm and a very irregular flow pattern in the larger collecting lymph vessels. They also pointed out that an increase in the pressure threshold for activation from upstream to downstream segments would facilitate the coordination of contractions between the segments and increase the efficiency of lymph transport. Such a threshold gradient was indeed confirmed experimentally by Hargens and Zweifach (1977). The finest collecting lymph vessels in the rat mesentery with a diameter of 30 – 40 μm showed an average threshold of 4 cmH_2O, compared with ~10 cmH_2O in vessels with diameters of 220 μm.

We evaluated the reasons why the regular and irregular spontaneous contractions were observed in isolated lymph vessel preparations, by using pumping preparations of collecting lymph vessels and transmural electrical stimulation [Ohhashi, 1987]. We demonstrated a very interesting finding that an activation of noradrenergic sympathetic nerve fibers innervated into the wall of lymph vessel caused a clear movement of the pacemaker site of the regular spontaneous contractions with peristalsis-type, resulting in appearance of the irregular spontaneous contraction with pendular-type. Thus the collecting lymph vessels about 5 cm long and 1 – 3 mm in diameter were dissected from fresh bovine mesentery, cannulated at both ends, and set up in Krebs-bicarbonate solution in a horizontal organ bath so that spontaneous contractions of the vessel produced propulsion of intravascular fluid. The outflow pressure and outer diameter of the lymph vessel at the pacemaker site of the contractions were simultaneously measured by a pressure transducer and a hand-made new diameter gauge with image sensor [Sakaguchi et al., 1979]. The platinum electrode was adjusted at the pacemaker site in order to stimulate selectively noradrenergic nerve fibers innervated on the pacemaker cells. Figure 2 shows representative responses of two kinds of pumping lymphatic preparations to the electrical stimulation which are rectangular pulses of 50 V, 0.5 ms, and 2 Hz. As shown in the right panel, in almost preparations the electrical stimulation of the pacemaker site, which is located in the

wall in the immediate vicinity of the inlet valve, caused the pacemaker site to move to the intervalvular region of the lymphangion (P' in the panel). The spontaneous contractions with the new pacemaker site, resulting in the contractions with pendular-type, produced passive distension of the outer diameter at the valvular region. About 1 min after an interruption of the stimulation, the moved pacemaker site returned to the previous one, valvular region. On the other hand, as shown in the left panel, in some preparations (example for increasing the environmental temperature) the pacemaker site of the spontaneous contractions is located at the middle portion of the lymphangion. In that case, the electrical stimulation produced an increase of the frequency of the contractions only, but not move the pacemaker site. The findings suggest that the regulatory action of noradrenergic nerve fibers on the lymphatic pump activity may be depended upon the position of pacemaker site of the spontaneous contractions, resulting in the appearance of peristalsis- or pendular-type contractions.

What physiological significance does exist there in the irregular pendular-type spontaneous contractions involved in several lymphangions? One of the possible means may be related to absorb the fluid and protein through lymphatic capillaries, providing an increase of lymph formation. In fact, Benoit et al. (1991) demonstrated that lymphatic pumping accounted for the majority of increase of lymph formation by less than five times control.

2.3 Rho-Rho kinase pathway in spontaneous contractions

It is well known that the myogenic tone of smooth muscles in collecting lymph vessels regulates elastic behavior of the wall and then results in the regulation of rhythm and amplitude of the spontaneous contractions. In addition, an increase Ca^{2+} influx through the membrane and release of membrane-bound and intracellular stored Ca^{2+} are also known to contribute the agonist-mediated contractions of lymphatic smooth muscles [Azuma et al., 1983; Ohhashi et al., 1980].

Recent studies have revealed that the important roles for small GTPase Rho and its effector Rho-associated kinase (Rho kinase) in Ca^{2+}-independent regulation of smooth muscle contractions. The Rho-Rho kinase pathway modulates the level of phosphorylation of the myosin light chain of myosin II, mainly through the inhibition of myosin phophatase, and contributes to agonist-induced sensitization in smooth muscle contractions [Fukata et al., 2001]. In addition, in the agonist-induced contractions of vascular smooth muscles, the Rho-Rho kinase pathway is also directly involved in phosphorylating myosin light chain, resulting in augmentation of

contraction [Kureishi et al., 1997].

Little information exists, however, regarding the crucial roles of the Rho-Rho kinase pathway in the regulation of spontaneous contractions of lymph vessels. Thus we examined the effects of Y-27632, a selective Rho kinase inhibitor [Fukata et al., 2001] and okadaic acid, a selective myosin phophatase inhibitor [Gong et al., 1992] on the spontaneous contractions of isolated iliac rat lymph vessels.

Figure 3A shows representative recording of Y-27632 (1, 3, and 6 μM)-induced responses of the spontaneous contractions in the lymph vessels with intact endothelium. Y-27632 induced a dose-related dilation of the lymph vessels with cessation of the spontaneous contractions. The Y-27632-induced period of cessation increased dose-dependently. Figure 3, B-D summarizes the effects of Y-27632 on %Dmax, %Dmin, and percent frequency, respectively of the spontaneous contractions in the lymph vessels (n=8). Y-27632 significantly increased the %Dmax. The Y-27632-induced maximum dilations were independent of the concentration of agonist used. On the other hand, the %Dmin of the lymph vessels increased dose-dependently. Y-27632 significantly decreased the percent frequency of the spontaneous contractions of the lymph vessels. Figure 3E summarizes the periods of cessation of the spontaneous contractions. The lymph vessels without intact endothelium show a significant dilation with cessation of the spontaneous contractions in response to the same concentration of Y-27632, as well as these obtained with the lymph vessels with intact endothelium. Okadaic acid significantly constructed the lymph vessels and reduced the frequency of the spontaneous contractions. These findings suggest that Rho-kinase and myosin phosphatase activity in the lymphatic smooth muscles may contribute the regulation of rhythmic pump activity in lymph vessels [Hosaka et al., 2003].

3.Lymphatic function and oxygen tension in lymph

3.1 Oxygen gradients in the microcirculation

It seems likely that some loss of oxygen from the blood begins as soon as the blood exists the lung [Tsai et al., 2003]. However, the amount of oxygen lost is apparently not significant until blood reaches the smallest arteries since the oxygen level in the large arterioles is similar to that in these vessels [Vovenko, 1999]. This loss of oxygen accelerates in the arteriolar network as documented by techniques that measure periarteriolar PO_2 , HbO_2 saturation, and intravascular PO_2.

The longitudinal gradients of oxygen tension in the capillary network vary

greatly depending on the vascular bed studied. Duling and Berne (1970) noted that when the hamster was breathing room air the perivascular PO_2 values measured with the microelectrode technique in the cheek pouch indicated that the blood was 67 % desaturated upon entering the capillaries. Since venous blood PO_2 was reported to be higher (36 mmHg), it may be hypothesized that in this tissue, capillary PO_2 represents the lowest intravascular PO_2 value. A study in the awake hamster model [Kerger et al., 1995] using the phosphorescence technique similarly reported a small fall in PO_2 between terminal arterioles and venules, indicating an approximately constant capillary PO_2 of ~30 mmHg. Also it was found in this preparation that fourth-order arteriolar PO_2 was 34 ± 2 mmHg and tissue PO_2 between the capillaries was 25 ± 6 mmHg, showing that tissue and capillary PO_2 is relatively uniform in this preparation. These data show that in this unanesthetized hamster model the whole microvascular network participates in tissue oxygenation and that the capillaries do not play a major role in the process [Tsai et al., 2002].

In contrast, data on longitudinal gradients in the venular network for a number of the studies show that the gradual rise in PO_2 is notable for most vascular bed except for the venular networks of the hamster skinfold and bat spinotrapezius muscle where there is a substantial rise. Thus, Pittman and Duling (1975) found that larger venules in the hamster cheek pouch preparations had greater mean saturation than small venules. In the study of Kerger et al. (1995), in the awaked hamster skinfold window preparation using the phosphorescence technique, venular capillaries and venules had respective PO_2 values of 30 ± 10 mmHg. Shonat and Johnson (1997) also studied the oxygen tension in venous microcirculation of rat spinotrapezius muscle utilizing the phosphorescence decay technique. They found that the mean intravascular PO_2 levels in postcapillary venules (diameter; 11± 4 μm), venular vessel (diameter; 31 ± 9 μm), and arcading venules (diameter; 60 ± 22 μm) rose sequentially from 22 ± 9 to 26 ± 10 to 33 ± 8 mmHg, respectively. Thus the findings suggest that there exists an increasing mean oxygen level in progressing downstream this venular microcirculation.

3.2 Oxygen gradients in the lymphatic system

The presence of a significant perimicrovascular oxygen gradient [Tsai et al., 2002] determines that tissue PO_2 should always be significantly lower than capillary blood PO_2 and therefore also venular and venous blood PO_2. This concept has been verified by measurements of tissue PO_2 with phosphorescence technique. It is noteworthy that this technique, which reveals significant oxygen gradients in the

perimicrovascular tissue, tends to show a relatively uniform PO_2 environment [Tsai et al., 2002]. By measuring of oxygen tensions (PO_2) of blood and lymph with a modified Clark needle oxygen electrode, Bergofsky et al. (1962) proposed that a better estimate of tissue PO_2 could be obtained by measuring PO_2 of excess tissue fluid (lymph) that returns to the circulation via the lymphatic system. They revealed that marked differences existed between the gaseous composition of lymph and blood; the PCO_2 of lymph averaged 5 ± 3 mmHg higher than venous blood. On the other hand, the oxygen tension of lymph differed markedly from the PO_2 of blood; whereas the average PO_2 of arterial blood was 80 mmHg and that of venous blood 42 mmHg, the PO_2 of lymph averaged only 8 ± 6 mmHg.

This concept was reevaluated using polarographic oxygen electrodes by Barankay et al. (1976) in the lymph vessels of the rabbit hindlimb and by Farrell et al. (1979) in the mesenteric lymph vessels of the dogs. The microelectrode studies were carried out in relatively large lymph vessels, yielding an average lymph PO_2 of 28 and ~ 50 mmHg in the hindlimb and mesentery, respectively. They concluded that PO_2 values of the fluid in collecting lymph vessels and thoracic ducts were not representative of tissue PO_2. Recently Hangai-Hoger et al. (2004) attempted to measure the PO_2 of lymph in mesenteric lymph vessels (mean diameter, 43.6 μm) of anesthetized rats by using oxygen phosphorescence quenching technique. They also confirmed that the lymph and perilymphatic adipose tissue PO_2 were 20.6 ± 9.1 and 34.1 ± 7.8 mmHg, respectively

We also investigated the PO_2 of lymph through the thoracic ducts in anesthetized dogs by using an oxygen electrode. We also examined the effects of 3 molar potassium chloride-mediated cardiac arrest on changes in the flow rate of lymph and PO_2 value of the lymph [Ikomi et al., 2002]. Thus the mongrel dogs were anesthetized with sodium pentobarbital (30 mg/Kg, i.v.) and ventilated artificially using a respirator with room air. The thoracic duct was cannulated at the cervical position of the vessel with a polyethylene catheter equipped with a needle-typed oxygen electrode. The outer end of the catheter was attached with a domestic-made drop counter flow meter that kept fixed at the same position as that of the heart at the hydrostatic pressure level. With the flow meter, the changes in the flow rate of lymph were measured continuously. The femoral artery and vein were also cannulated with polyethylene catheters to measure changes in systemic arterial pressure and administrate physiological saline solution at a constant rate of 100 ml/hr during the experiment. Figure 4 shows representative recordings of changes in the

PO_2 of lymph, the flow rate of lymph, and systemic arterial pressure before and after the administration of 3 M KCl in an anesthetized dog. The PO_2 of lymph at physiological condition was around 35 mmHg in the anesthetized dog. An intravenous administration of 3 M KCl produced a rapid and large reduction of the arterial pressure and then resulted in cardiac arrest of the dog. The cardiac arrest caused a transient increase of lymph flow rate that was kept during ~ 15 min after the cardiac arrest. It also produced a gradual decrease of the PO_2 in the lymph, which became stable, ~10 mmHg, at 15min after the cardiac arrest.

In conclusion, there is a significant longitudinal gradient of PO_2. Thus mean PO_2 levels in lymphatic capillaries, collecting lymph vessels, and thoracic ducts rose sequentially from ~ 8, ~ 20, and ~ 35 mmHg, respectively. Therefore it should be emphasized that the lymphatic endothelial cells seem to have physiological functions under the specific environment of lower oxygen tension of 8 ~ 35 mmHg.

3.3 Interstitial oxygen gradients in solid tumors

Oxygen and pH are key environmental factors in the development and growth of tumors and their response to treatment [Helmlinger et al., 1977; Jain, 1977]. Oxygen and pH levels affect tumor cell metabolism, glucose and oxygen consumption rates, and tumor cell proliferation and viability [Vaupel et al., 1989; Gullino et al., 1967; Casciari et al., 1992]. Hypoxia can stimulate angiogenesis [Shweiki et al., 1992]. Hypoxia can also induce tumor apoptosis [Shimizu et al., 1996], as well as select for cells defects on apoptosis [Graeber et al., 1996], thereby affecting tumor growth. Tumor cell migration and immune cell response are other key processes in tumor biology that may be influenced by the levels of pH and oxygen in the extracellular milieu [Krtolica and Ludlow, 1996; Loeffler et al., 1992]. The oxygen and pH also play important roles in the response of tumors to radiation, chemotherapy, hyperthermia and photodynamic therapy [Helmlinger et al., 1997].

The temporal and spatial heterogeneities in blood flow are expected to lead to a compromised metabolic microenvironment in tumors. However both pH and PO_2 decrease as distance from the tumor-mediated blood capillary increase, leading to acidic and hypoxic regions in tumors. Thus the lowest PO_2 and pH values in tumor tissues become ~ 5 mmHg and 6.6 ~ 6.7, respectively [Jain, 1977].

3.4 Oxygen tension in lymph and spontaneous contractions

It is noteworthy that there exist lots of vasa vasorum within the media of collecting lymph vessels with spontaneous contractions [Ohhashi et al., 1977], which may be essential for maintaining the vigorous contractions of lymphatic smooth

muscles because of the above-mentioned lower oxygen tension in lymph. Thus smooth muscles in bovine mesenteric collecting lymph vessels are well-developed and arranged in three layers; namely, the internal longitudinal, intermediate circumferential, and external longitudinal. The outer longitudinal layer is much thicker than the other two layers. There are few elastic fibers but a large number of collagen fibers underneath the endothelial cell lining and among the smooth muscle layers. A large number of mitochondria, gathered in a cluster, are seen on both sides of the nucleus along the longitudinal cell axis of the smooth muscle cells. Numerous glycogen granules were found among and around the mitochondria. These structural features might be a morphological manifestation of the high metabolic activity required for spontaneous contractions of the lymphatic smooth muscles [Ohhashi et al., 1977].

Figure 5 shows a transverse section of blood capillary found within the external longitudinal smooth muscle layer. Because It was identified as blood capillary by the presence of complete basement membrane and the configuration of endothelium. Occasionally, erythrocytes were found in the lumen. The presence of vasa vasorum within the media may reflect a relative high oxygen requirement of the lymphatic smooth muscle cells and the relatively low oxygen supply from the lymph flowing through the lymph vessel. An ample supply of oxygen will be required to maintain the spontaneous contractions in the collecting lymph vessel.

3.5 K_{ATP} channels and spontaneous contractions

ATP-sensitive K^+ channels (K_{ATP}), which were first found in cardiac muscle [Noma, 1983], are located in the plasma membrane of cells including vascular and non-vascular smooth muscle cells [Quayle et al., 1997] and participate in the regulation of the membrane potential. The intracellular concentration of ATP is a determinant to activate and deactivate K_{ATP} channels. Thus ATP produced by respiratory activity and metabolic demand in the cells may contribute to feedback mechanisms that control cell functions through an activation of K_{ATP} channels.

On the other hand, as mentioned previously, the lymphatic smooth muscles demonstrate clearly spontaneous contractions, and contain numerous mitochondria and glycogen granules. The existence of vasa vasorum within and among the smooth muscle layers was confirmed. The functional and morphological properties suggest that a lot of ATP in the smooth muscle cells seem to be produced in the collecting lymph vessels with spontaneous contractions. We hypothesized that the K_{ATP} channels may play an important role in the regulation of spontaneous

contractions in the collecting lymph vessels.

Thus we investigated physiological roles of the K_{ATP} channels for spontaneous contractions in isolated rat mesenteric lymph vessels (diameter, 80 - 150 μm) [Mizuno et al., 1999]. Pinacidil (a K^+ channel opener) inhibited the lymphatic spontaneous activity. In the presence of glibenclamide (a selective K_{ATP} channel blocker), the pinacidil-mediated inhibition of the spontaneous contractions in the lymph vessels was significantly reversed. Glibenclamide itself, however, did not affect the frequency of the contractions. Thus it has been suggested that the K_{ATP} channels are involved in the regulation of spontaneous contractions in isolated rat collecting lymph vessels.

von der Weid (1998) also demonstrated that the K_{ATP} channels play a central role in lymphatic smooth muscle hyperpolarization produced by a nitric oxide (NO)-mediated increase of cyclic GMP synthesis, as well as by β-adrenoceptor-induced production of cyclic AMP. Both pathway produced the opening of the K_{ATP} channels through the activation of protein kinase.

4. Cultures of lymphatic endothelial cells (LECs)

4.1 Cultures of LECs in dogs, sheep, and cattle

The establishment of endothelial cell lines isolated from blood vessels have provided much information on the culture methods, growth requirements, and biochemical processes of the blood vascular endothelium [Gimbrone, 1976; Goldsmith et al., 1984; Jaffe, 1984; Piovella et al., 1978; Schwartz, 1978; Wagner et al., 1982; Gordon et al., 1983]. These in vitro studies have yielded a considerable body of information regarding the function of the blood vascular endothelial cells and they also provide a valuable tool for further investigations into the role of the endothelium in normal and pathologic processes.

On the other hand, beginning with the studies of Johnston and Walker (1984), methods are now being developed for both primary [Gnepp and Chandler, 1985; Jones and Yong, 1987] and long-term [Leak and Jones, 1993] culture of lymphatic endothelial cells. Using collagenase, trypsin-EDTA, and treatment with 5 % CO_2 and 95 % air in a humidified incubator, Leak and Jones (1993) have been able to successfully isolate and grow primary cultures of the lymphatic endothelium of bovine mesenteric collecting lymph vessels, which were subcultured, frozen for storage, subsequently thawed with good recovery and growth, and serially subcultured. The morphological features of cultured lymphatic endothelial cells were consistent with those observed for the endothelium of intact lymphatic vessels. A prominent

feature of growing culture was the appearance of large vacuoles in the perinuclear region of the cytoplasm, which became filled with fluid and cell debris engulfed from the culture medium. The basal cell surface lacked well-defined basal lamina and anchoring filaments was observed extensively from the basal plasmalemmal surface into the underlying substratum. The lymphatic endothelial cells in cultures were positive for Factor VIII-related antigen. However, specific granules, characteristic of Weibel-Palade bodies were not observed in ultrathin sections of confluent cultures. F-actin was identified in the culture of lymphatic endothelial cells using fluorescein phalloidin, and in confluent cultures actin filaments were located at the periphery of the cell as a continuous circumferential part of the cell. By using heparin and endothelial cell growth supplement in the culture medium, they have been able to grow stable cultures of lymphatic endothelial cells and could been maintained when serially subcultured for over two years.

Using collagenase treatment with 5 % CO_2 and 95 % air in a humidified incubator, we (1999) isolated successfully and grew primary culture of lymphatic endothelial cells of canine thoracic ducts. The cultured cells were also positive for Factor VIII-related antigen and able to uptake of Dil-Ac-LDL. As shown in Figure 6, the immuno-reactivities to anti-endothelial constitutive NO synthase (ecNOS) and anti inducible NOS (iNOS) were positive significantly on the cultured lymphatic endothelial cells. When we stained 14 samples of the cultured cells to anti-ecNOS, the immunoreactive signal was intense in the intranuclei and cytoplasma (10 out of 14). In 4 out of 14 samples, the intense signal of anti-ecNOS was restricted in the nuclei. In contrast, the immunoreactivity of anti-iNOS to the cultured cells was not so intense, but clearly positive at the cytoplasma of the lymphatic endothelial cells. These immunoreactions were abolished by pre-absorption with the relevant blocking peptide. In compatible with our findings, Marchetti et al. (1997) also showed a marked immunoreactivities of ecNOS and endothelin in the cultured lymphatic endothelial cells of bovine mesenteric collecting lymph vessels. Leak et al. (1995) also demonstrated clearly that lipopolysaccharide of Escherichia coli (LPS) stimulated the induction of iNOS in the lymphatic endothelial cells of bovine and sheep mesenteric collecting lymph vessels, exhibiting an intense staining reaction for the anti-iNOS antibody in the nucleus and cytoplasma. The findings for the localization of iNOS in the lymphatic endothelial cells presented by us (1999) may be strongly agreed with the data of the LPS-mediated induction of iNOS in the cultured endothelial cells [Leak et al., 1995]. We used 20 % fetal bovine serum for the cultures, which seemed to

contain various cytokines, resulting in the induction of iNOS in the cultured lymphatic endothelial cells [Fig. 6].

4.2 $VEGF_{165}$-mediated generation of NO in LECs

By using the LECs of canine thoracic ducts, we investigated the effects of vascular endothelial growth factor165 ($VEGF_{165}$) on the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$)-transients and mechanical activity of isolated canine thoracic ducts. The $[Ca^{2+}]_i$-transients were evaluated using fluorescence probes, fluo 3 acetoxymethyl ester (AM) and fura red-AM. The dye probes were loaded into the LECs with MOPS-buffered physiological saline solution containing 0.025 % cremophor for 60 min at 37 °C in the dark. Fluorescence images of $[Ca^{2+}]_i$-transients were obtained with a laser-scanned confocal microscope. The fluorescein filter (488 nm) was used to excite the fluo 3 and fura red. Thus when excited at 488 nm, the fluo 3 exhibits an increase in green, which suggests the fluorescence on Ca^{2+} binding, whereas the fura red shows a decrease in red fluorescence (640 nm). The emission ratio of fluo 3 to fura red was then calculated as an index for the relative changes in the $[Ca^{2+}]_i$ of the LECs. $VEGF_{165}$ (0.1, 1.0, and 10 ng/ml) caused a rapid increase of the $[Ca^{2+}]_i$. The time course of the $[Ca^{2+}]_i$ –transients was depended upon the dose of $VEGF_{165}$ used. Thus 10 ng/ml $VEGF_{165}$-induced increase of the $[Ca^{2+}]_i$ was kept during ~ 160 s. In contrast, 0.1 ng/ml $VEGF_{165}$-induced increase of the $[Ca^{2+}]_i$ was transient and then returned rapidly to the control. Pretreatment with 10^{-5} M genistein or 5 x 10^{-6} M herbimycin A produced a significant reduction of the $VEGF_{165}$-mediated rapid increase of the $[Ca^{2+}]_i$. In the presence of 10^{-6} M thapsigargin, $VEGF_{165}$ caused no significant effect on the $[Ca^{2+}]_i$ –transients. Pretreatment with Ca^{2+}-free solution containing 0.1 mM EGTA produced no significant effect on the $VEGF_{165}$ –mediated peak increase of the $[Ca^{2+}]_i$, but significantly suppressed the sustained part of the $[Ca^{2+}]_i$–transients produced by 10 ng/ml $VEGF_{165}$.

The $VEGF_{165}$ (0.1 ~ 10 ng/ml) caused a significant dilation of the bioassay preparations of isolated canine thoracic ducts with intact endothelium, which were precontracted with U 46619. The $VEGF_{165}$-mediated dilation was significantly reduced by treatment with 3 x 10^{-5} M L-NAME. The action of L-NAME was suppressed significantly by the simultaneous administration of 10^{-3} M L-arginine. Mechanical rubbing of the endothelium caused significant reduction of the $VEGF_{165}$-mediated dilation. These finding suggest that $VEGF_{165}$ may activate the receptor-related tyrosine kinase and release the $[Ca^{2+}]_i$ from inositol 1,4,5

triphosphate-sensitive intracellular Ca^{2+} stores within the LECs. The release of Ca^{2+} within the LECs may activate ecNOS and then generation of endogenous NO, which results in a significant dilation of the bioassay lymphatic preparations.

4.3 Histamine-mediated generation of NO in LECs

In the cultured LECs, as shown in Figure 7, 10^{-5} M histamine caused rapid and sustained increases of the $[Ca^{2+}]_i$ of the cultured LECs of canine thoracic ducts. Figure 7 shows representative images of the histamine-mediated changes in the $[Ca^{2+}]_i$ of the cultured LECs, demonstrated with pseudo-color images of the $[Ca^{2+}]_i$ (low concentration; blue, ----, yellow, and red ; high concentration), before and 40 s after an administration of the agonist. The supernatant of cultures of the LECs obtained 1 min after an administration of 10^{-5} M histamine caused a significant relaxation of the bioassay preparation of canine thoracic duct precontracted with U 46619 in the presence of H_1- and H_2-antagonists. The supernatant-mediated relaxation of the bioassay preparations was significantly reduced by pretreatment with 3 x 10^{-5} M L-NAME into the culture media of the LECs. The L-NAME-mediated reduction of the relaxation was significantly reversed by additional treatment with 10^{-3} M L-arginine into the culture medium. These findings suggest that histamine produces a significant increase of the $[Ca^{2+}]_i$ of the LECs, which activates the ecNOS of the LECs, generates endogenous NO within the LECs, and results in relaxation of the lymphatic smooth muscle cells [Ohhashi et al., 1996].

4.4 Hypoxic cultures of LECs in rats

The cultured lymphatic endothelial cells of dogs, sheep, and cattle have been used to investigate biological and morphological properties of the lymphatic endothelial cells [Johnston and Walker, 1984; Gnepp and Chandler, 1985; Jones and Young, 1987; Leak and Jones, 1993; Nojiri et al., 1999]. No report, however, exists regarding the establishment of a lymphatic endothelial cell line isolated from small experimental animals such as rats and mice. The establishment of a rat or murine lymphatic endothelial cell line still remains unsuccessful. If established, key studies of cellular and molecular mechanisms of tumor-mediated lymphangiogenesis or embryological development of lymph vessels and lymph nodes will be facilitated quickly. Recently we successfully isolated rat lymphatic endothelial cells by using enzymatic digestion with trypsin and the established the rat cell line by using EGM-2 as culture medium and hypoxic atmosphere (5 % CO_2, 5 % O_2 and 90 % N_2). The choice of the hypoxic atmosphere was related to the previously mentioned evidence that the lymphatic endothelial cells works physiologically at low atmospheric oxygen

levels ranging from 8 to 35 mmHg. Thus in the primary cultures on 14-th day, the number of the cultured lymphatic endothelial cells in the 5 % O_2 atmosphere was significantly larger than that obtained in the 21 % O_2 atmosphere. The mean number of the cultured cells in the 5 % O_2 and 21 % O_2 atmosphere were 48,750 ± 10,594 cells/ml and 4,333 ± 1,377 cells/ml, respectively [Mizuno et al., 2003]. Figure 8A shows a representative microphotographic phase-contrast image of the cultured cells in the low-oxygen atmosphere (~5 % O_2). As the cells became confluence, they demonstrated a monolayer with a uniform cobblestone appearance. The cellular shapes were usually polygonal, and each cell was contracted. Figure 8B demonstrates representative images of the arrangement of F-actin in cultured cells under hypoxic conditions. In the confluent cultures, F-actin filaments usually distributed on the plasma membrane of the cultured cells as circumferential thin bundles and were distributed in the cytoplasma as filamentous bundles. The immunofluorescence studies demonstrated that the Factor VIII-related antigen was found in the perinuclear region of the cultured cells in the low oxygen atmosphere. The cell surface carbohydrates (D-galactose-α and D-N-acetylgalactosamine α) was also observed in the cells cultured under the low-oxygen conditions. The cells also demonstrated marked phagocytosis of Dil-Ac-LDL in the perinuclear region. These finding suggest that the cultured cells may be identified as rat lymphatic endothelial cells. The choice of endothelial growth medium (EGM)-2 as culture medium and the hypoxic atmosphere (~5 % O_2) enabled us to culture successfully the rat endothelial cells.

Different and common properties between cultured arterial endothelial cells (ECs) and cultured lymphatic ECs (rat) are summarized in Table 1.

5. NO and oxygen radicals in the lymphatic system

As high concentrations, free radicals and radical-derived non-radical reactive species are hazardous for living organisms and damage all major cellular constituents. At moderate concentrations, however, nitric oxide (NO), superoxide anion, and related reactive oxygen species (ROS) play an important role as regulatory mediators in signaling processes [Lum and Roebuck, 2001; Cai and Harrison, 2000; Dröge, 2002; Beckman and Koppenol, 1996]. Many of the ROS-mediated responses actually protect the cells against oxidative stress and reestablish “redox homeostasis”. Higher organisms, however, have evolved the use of NO and ROS also as signaling molecules for other physiological functions. These include regulation of vascular tone, monitoring of oxygen tension in the control

of ventilation and erythropoietin production, and signal transduction from membrane receptors in various physiological processes. NO and ROS are typically generated in these cases by tightly regulated enzymes such as NOS and NAD(P)H oxidase isoforms, respectively. In a given signaling protein, oxidative attack induces either a loss of function, a gain of function, or a switch to a different function. Excessive amounts of ROS may arise either from excessive stimulation of NAD(P)H oxidases or from less well-regulated sources such as the mitochondrial electron transport chain [Dröge, 2002]. In mitochondria, ROS are generated as undesirable side products of the oxidative energy metabolism. As excessive and/or sustained increase in ROS production has been implicated in the pathogenesis of cancer, diabetes mellitus, atherosclerosis, ischemia/reperfusion injury and other diseases [Dröge, 2002].

NO contrasts with most intercellular messengers because it diffuses rapidly and isotropically through most tissues with little reaction but can not be transported through the vasculature due to rapid destruction by oxy-hemoglobin. The rapid diffusion of NO between cells allows it to locally integrate the responses of blood vessels to turbulence, and modulate synaptic plasticity in nervous [Beckman and Koppenol, 1996]. NO is not necessarily short-lived and is intrinsically no more reactive than oxygen. The reactivity of NO per se has been greatly overestimated in vitro, because no drain is provided to remove NO. NO persists in solution for several minutes in micro-molar concentrations before it reacts with oxygen to form much stronger oxidants like nitrogen dioxide. NO is removed within seconds in vivo by diffusion over 100 μm through tissues to enter red blood cells and react with oxy-hemoglobin. The direct toxicity of NO is modest but is greatly enhanced by reacting with superoxide to form peroxynitrite ($ONOO^-$). NO is the only biological molecule produced in high enough concentrations to out-compete superoxide dismutase for superoxide. Peroxynitrite reacts relatively slowly with most biological molecules making peroxynitrite, a selective oxidant. Peroxynitrite modifies tyrosine proteins to create nitro-tyrosines, leaving a footprint detectable in vivo.

Oxygen is an important regulator of microvascular tone throughout most vascular beds in many species [Nase et al., 2002]. The study of the role of oxygen in microvascular regulation has been greatly implicated by the finding that reduced oxygen availability can increase the release of endothelium-derived NO [Busse et al., 1983; Pohl and Busse, 1989]. Manevich et al. (2001) and Wei et al. (2001) also demonstrated an 80 % increase in NO generation during acute oxygen deprivation and the NO response was evident within 15 s after decreased oxygen availability.

Various investigators have also demonstrated that hyperpolarizing factors [Fredrick et al., 1994], cyclo-oxygenase products [Messina et al., 1992], and adenosine [Marshall, 2000; Park et al., 1992] can stimulate endothelial cells during oxygen deprivation. Recently Nase et al. (2002) demonstrated that in rat intestine, reduced oxygen availability increased both arteriolar and venular NO and that the main site of NO release under these conditions was from endothelial cells.

Taken into consideration of the above-mentioned backgrounds of NO and ROS, it may be reasonable to expect that NO and ROS may play important roles in the regulation of the spontaneous contractions-mediated lymph transport in physiological or pathophysiological conditions. In fact the dense immunoreactivity of ecNOS and iNOS were confirmed in the cultured canine lymphatic endothelial cells [Nojiri et al., 1999] and in bovine mesenteric lymph vessels in vivo [Marchetti et al., 1997].

5.1 NO inhibits spontaneous contractions in vitro

Lymphatic endothelial cells, as well as arterial and venous endothelial cells, have the potential generation of endogenous NO [Ohhashi and Takahashi, 1991; Mizuno et al., 1998; von der Weid et al., 2001].

In precontracted canine thoracic ducts with intact endothelium, acetylcholine (ACh) produced dose-related relaxations. The relaxations seem to be mediated via high-affinity muscarinic receptor subtype, because they were competitively antagonized by atropine, demonstrating a pA_2 value of 10.4 in the Arunlakshana and Schild analysis (1959). In contrast, in isolated rings of rabbit thoracic aorta and canine femoral artery the ACh-mediated endothelium-dependent relaxations are produced by a low-affinity muscarinic receptor subtype only (pA_2: 8.4 – 8.8) [Eglen and Whiting, 1985]. Thus, it may be a characteristic feature of lymph vessels that the high-affinity muscarinic subtype is related to the ACh-induced endothelium-dependent relaxation. Mechanical rubbing of the endothelium significantly reduced the ACh-induced relaxation. Pretreatment with aspirin, an inhibitor of cyclo-oxygenase, did not affect the ACh-mediated relaxation, suggesting that prostacyclin and the other vasodilative prostaglandins did not play a pivotal role in the ACh-mediated relaxation of the thoracic ducts. On the other hand, oxyhemoglobin (an inhibitor of NO) [Martin et al., 1985], L-NMMA (an inhibitor of NO biosynthesis) [Watanabe et al., 1988], and methylene blue (an inhibitor of guanylate cyclase) [Ignarro and Kadowitz, 1985] markedly suppressed the ACh-mediated relaxation in canine thoracic ducts with intact endothelium. ACh also produced a marked relaxation in the so-called sandwich-mounted preparation, demonstrating that

the lymphatic endothelial cells of longitudinal strip in response to ACh must release some transferable substance(s) that, on diffusion into the ring segment, activated relaxation of the precontracted lymphatic smooth muscle cells. The findings strongly suggest that the ACh-induced relaxations are mainly mediated through the release of NO or its related compound(s) from the lymphatic endothelial cells and diffusion of the substance(s) in the wall of canine thoracic ducts. The substance(s) diffuses into the smooth muscle cells and then produces the accumulation of cellular guanosine 3', 5'-cyclic mono-phosphate (GMP), which results in the relaxation of canine thoracic ducts [Ohhashi and Takahashi, 1991].

Mizuno et al. (1998) also elucidated the nature of endothelium-derived factors, produced in basal conditions and in response to agonists, that affect the smooth muscle tone of cannulated with glass micro-pipettes and pressurized rat iliac lymph vessels. They concluded that endothelium NO and prostaglandins are important mediators of lymphatic vasomotion.

NO release from lymphatic endothelial cells is also known to be able to inhibit rhythm and amplitude of the rhythmic pump activity of isolated bovine mesenteric collecting lymph vessels [Yokoyama and Ohhashi, 1993]. Regular rhythmic pump activity at a constant rate of 2 – 4 beats/min were observed. ACh at concentrations between 10^{-7} and 10^{-6} M caused both negative chrono- and ino-tropic effects on the rhythmic pump activity. The ACh-induced negative chrono- and ino-tropic effects were significantly reduced when the intact endothelium of the lymph vessels was removed mechanically. The ACh-induced negative chrono- and ino-tropic effects were significantly reduced by pretreatment with 3×10^{-5} M L-NMMA. An additional treatment with 10^{-4} M L-arginine caused a complete reversal of the L-NMMA-mediated reduction of the ACh-induced both negative effects on the rhythmic pump activity. Endogenous NO liberating from the lymphatic endothelial cells seems to inhibit pacemaker activity of the rhythmic pump activity and reduce the myogenic conduction and/or the contractile ability of lymphatic smooth muscles. A marked increase of cytosolic 3', 5' cyclic GMP content in the lymphatic smooth muscle cells may also contribute to the NO-mediated negative chrono- and ino-tropic effects on the rhythmic pump activity in isolated bovine mesenteric collecting lymph vessels.

Atrial natriuretic peptides (ANP) also caused negative chrono- and ino-tropic effects on rhythmic pump activity in the isolated bovine mesenteric collecting lymph vessels through synthesis of 3', 5' cyclic GMP in the walls, independent of the

lymphatic endothelial cells [Ohhashi et al., 1990].

By using intracellular microelectrode recordings, von der Weid et al. (2001) attempted to determine whether NO affects the pacemaker events that initiate vasomotion in lymphatic vessels of the guinea pig mesentery. The pacemaker activity recorded as spontaneous transient depolarization (STDs) and is likely to arise through synchronized Ca^{2+} release from intracellular stores. It is clearly shown that ACh-induced endothelium-derived NO and exogenous NO released by SNP reduced the frequency and amplitude of the STDs. The reduction of STD frequency and amplitude was independent of the NO-mediated hyperpolarization of the smooth muscle. The SNP-induced reduction of SDP frequency and amplitude was abolished during superfusion with the soluble guanylate-cyclase inhibitor 1*H*-[1,2,4,] Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and diminished in the presence of cyclic GMP and cyclic-AMP-dependent protein kinase inhibitors. They proposed the hypothesis that NO inhibits vasomotion primarily by production of cyclic GMP and activation of both cyclic GMP and cyclic-AMP-dependent protein kinase, which reduce the size and frequency of STDs, probably by acting on the underlying synchronized Ca^{2+} release from intracellular stores.

5.2 NO inhibits lymphatic pump activity in vivo

Shirasawa et al. (2000) attempted to evaluate physiological roles of endogenous NO in lymphatic pump activities of rat mesenteries in vivo by using an intravital video-microscope system. Changes in the pumping frequency (F), the end-diastolic diameter (EDD), and the end-systolic diameter (ESD) of the mesenteric lymph microvessels were measured with the microscope system and then the pump flow index (PFI) was calculated. A 15-min superfusion of 30 μM L-NAME in the mesenteries caused a significant increase of F and PFI and a significant decrease of the EDD and ESD. Simultaneous superfusion of 1 mM L-arginine with 30 μM L-NAME produced a significant reversal of the L-NAME-mediated increase of F and decrease of ESD. A 15-min superfusion of 100 μM aminoguanidine caused no significant effect on F, EDD, and ESD of the mesenteric lymph vessels in vivo. They concluded that endogenous NO has physiologically modulated the lymphatic pump activity in rat mesentery in vivo and that the production and release of NO may be mediated by endothelial constitutive NOS but not by inducible NOS. The conclusion may be compatible with the studies obtained with the anesthetized sheep [Johnston, 1995].

5.3 Flow-mediated generation of NO and/or prostaglandins (PGs)

We also studied what physiological factor(s) contributes to the NO-dependent inhibition of lymphatic pump activity in vivo. Many previous studies showed that an increase in flow rate (in the presence of constant intraluminal pressure) increased the diameters of arterioles [Koller and Kaley, 1995] and venules [Dörnyei et al., 1997; Kuo et al., 1993] in an endothelium-dependent manner. Therefore we examined the effects of flow on lymphatic endothelial cells by using cascade arterial preparations without intact endothelium. The pressurized canine thoracic ducts were intraluminally perfused at a constant flow rate ranging from 0.5 to 2 ml/min. The flow rate of 2.0 ml/min produced ~ 30 % of SNP-induced maximal relaxation of the cascade bioassay preparations. The flow-mediated relaxation of the bioassay preparations was completely reduced by the mechanical rubbing of the lymphatic endothelial cells. Pretreatment with 5×10^{-5} M L-NAME on the lymphatic endothelial cells caused a significant reduction of the flow-mediated relaxation of the bioassay preparations. Pretreatment with 10^{-5} M indomethacin on the endothelial cells produced no significant effect on the flow-mediated relaxation. The authors suggested that the lymphatic endothelial cells can produce and release endogenous NO, but not vasodilative PGs, by the stimulation of flow (~2.0 ml/min). In addition a linear relationship was observed between the flow rate and the normalized amount of endogenous NO released from the lymphatic endothelial cells [Tsunemoto et al., 2003].

Gashev et al. (2002) also studied the effects of imposed flow on active lymph pumping under conditions of controlled intraluminal pressure. Rat mesenteric lymph vessels were isolated, cannulated and pressurized. Input and output pressures were adjusted to response various flow on the lymphatic endothelial cells. Lymphatic systolic and diastolic diameters were measured and used to determine contraction frequency and pump flow indices. Imposed flow inhibited the active lymph pumping in the mesenteric lymph vessels. Thus imposed flow reduced the frequency and amplitude of the rhythmic pumping. NO was partly but not completely responsible for the inhibitory action of flow on the mesenteric lymph pumping. Exposure to NO mimicked the effects of flow, and inhibition of the ecNOS by L-NMMA attenuated but did not completely reduce the inhibitory effects of Flow.

It is noteworthy that there are endothelin (ET)-B receptors on the plasma membrane of lymphatic endothelial cells in bovine mesenteric collecting lymph vessels. The stimulation of the ET-B receptors can release NO from the

endothelial cells, resulting in the negative chrono- and ino-tropic effects on the rhythmic pump activity [Sakai et al., 1999].

In contrast, Koller et al. (1999) demonstrated that in the isolated, pressurized rat iliac lymph vessels, the flow-induced decreases in the maximal and minimum diameters, and increase of oscillation frequency were eliminated by mechanical rubbing of lymphatic endothelium or pretreatment with indomethacin. Pretreatment with L-NAME did not affect the flow-mediated responses. They concluded that the sensitivity of lymphatic endothelium to changes in intraluminal flow could provide important intrinsic mechanisms for the regulation of lymphatic resistance by release of constrictor PGH_2 /thromboxane (TX)A_2.

In agreement with the finding, Gao et al. (1999) demonstrated that ATP caused a dose-related increase of vasomotion with endothelium-dependent mechanisms isolated guinea-pig mesenteric lymph vessels. The increase of vasomotion may be, in part, mediated with an increase of phospholipase A_2 and production of TXA_2 in the lymphatic endothelial cells. In fact, in the lymphatic system arachidonate metabolites, PGs, are well known to be one of key physiological substances in the regulation of spontaneous contractions of lymph vessels [Johnston and Gordon, 1981; Ohhashi and Azuma, 1984]. The evidence may be, in part, related to the previously mentioned concept that the functions in the lymphatic system work under the environment of lower oxygen tension and then under the environment the ROS including NO may play crucial roles in the regulation of the lymphatic functions.

5.4 Activated macrophage-mediated generation of NO and PGs

Recently, several biochemical studies demonstrated that bacterial lipopolysacchride (LPS) or interferon gamma (INF-γ) was able to induce both iNOS and cyclo-oxygenase (COX)-2 in macrophages [Akarasereenont et al., 1995; Misko et al., 1995]. It has also been shown that the extracellular space of wounds contains an exceedingly low concentration of L-arginine, attributable to the activity of macrophage-derived arginase [Albina et al., 1988]. Physiological roles for the macrophage arginase may be related to its superoxide production, phagocytosis, and protein synthesis in inflammation. In view of these findings, we [Wang et al., 1997; Wang, 1997] examined whether rat macrophage cultured with a very low concentration of L-arginine ($\sim$ 6 μM), a condition similar to the extracellular space of wounds, can induce and release both NO and vasodilative PGs. We also attempted to evaluate by using bioassay preparations the hypothesis that NO and vasodilative PGs released from the activated macrophages may contribute to the

regulation of mechanical activity of lymphatic smooth muscles. The supernatant of macrophages cultured in the L-arginine-free culture medium caused a significant reduction of the U 46619-mediated precontraction in the bioassay preparations without intact endothelium. The macrophage-induced vasodilation was significantly reduced by the co-culture of macrophages with 1 μM dexamethasone, 10 μM cycloheximide, 50 μM L-NAME, 10 μM indomethacin or 10 μM aspirin. The L-NAME-induced inhibition was significantly reversed by an additional treatment with 100 μM L-arginine. The co-culture with both L-NAME and indomethacin caused a significant reduction of the macrophage-induced vasodilation similar to the reduction produced by dexamethasone and cycloheximide. Co-culture with 10 μg/ml bacterial LPS caused a slight facilitation of the macrophage-induced vasodilation. We concluded that supernatant fluid of rat exuded macrophages cultured with low concentration of L-arginine causes an endogenous NO and vasodilative PGs-dependent relaxation of lymphatic smooth muscles isolated from canine thoracic ducts.

5.5 5-Hydroxytryptamine-mediated generation of NO in lymph nodes

Lymph from its origin in the lymphatic capillaries to its termination in the blood stream, usually passes through one or more lymph nodes [Yoffey and Courtice, 1970]. The supporting framework of lymph nodes is provided by two structures. Thus a capsule formed from trabeculae, which penetrate the node. Another fibrocellular net or reticulum, which spans the region between the capsules and trabeculae of lymph nodes in many species including human beings, monkeys, cattle, sheep and dogs [Yoffey and Courtice, 1970]. The lymph node capsules and trabeculae have a noradrenergic innervation that modulates their tone of smooth muscles via an activation of α-adrenoceptors [Thornbury et al., 1990], resulting in the regulation of release of lymphocytes from the regional lymph nodes [Felten et al., 1984].

We also demonstrated that 5-HT_1-like receptors exist in the monkey popliteal lymph nodes. The stimulation of the receptors produces an endogenous NO-dependent relaxation of the smooth muscles in the node through an activation of cytosolic guanylate cyclase in the cells [Mizuno and Ohhashi, 1995].

5.6 Reactive oxygen radicals (ROS) inhibit lymphatic pump activity in vivo

Zawieja et al. (1991) investigated the effects of oxygen-derived free radicals on the contractile activity of the mesenteric collecting lymph vessels in the anesthetized rats. Lymphatic rhythmic contractions were monitored before, during and after the

application of oxygen radicals. Contraction frequency (F), stroke volume (SV), ejection fraction (EF), contraction propagation (CP), and lymph pump flow (LPF) were determined from the lymphatic diameter tracings. Oxygen radicals were generated using hypoxanthine and xanthine oxidase. Exposure to oxygen radicals inhibited the lymphatic pumping mechanism; F fell from 15.5 ± 0.8 to 0.8 ± 0.7 beats/min. EF went from 0.44 ± 0.02 to 0.08 ± 0.04. CP dropped from 92 ± 2 to 56 ± 8 %. LPF fell precipitously from 41.0 ± 5.2 to 0.7 ± 0.4 nl/min. The effects of oxygen radicals were attenuated by superoxide dismutase, implicating superoxide anion as one of the predominant causative agents. They concluded that oxygen radicals significantly have inhibited the lymph pumping and that this inhibition could be a factor contributing to be formation of interstitial edema during inflammation.

We [Zhang et al., 1997] also evaluated the crucial roles of ROS in the regulation of lymph transport by studying the effects of photo-activation of fluorescein 5'-isothiocyanate (FITC)-dextran on rhythmic pump activity of rat mesenteric collecting lymph vessels in vivo. Rats were anesthetized with intraperitoneal administration of α-chloralose and urethane, and the mesenteries were studied by using intravital video-microscopic technique. The diameter of the collecting lymph vessels were continuously monitored and lymphatic pump parameters (end diastolic and end systolic diameters, stroke volume index, ejection fraction, contraction frequency, and pump flow index) calculated by FITC-dextran without illuminating, caused no disturbance of lymphatic pump activity. Photo-activated FITC-dextran significantly increased end systolic diameter and decreased stroke volume index, ejection fraction, contraction frequency, and pump flow index. End diastolic diameter was not changed throughout the experiments. Superoxide dismutase (120 U/ml) or catalase (5000 U/ml) had no protective effect on the photo-activated FITC-induced pump dysfunction, while histidine (a single oxygen quencher, 10 mM) significantly prevented the disturbance of pump parameters. Thus the findings suggest that photo-activation of FITC induced negative chrono- and ino-tropic effects on lymphatic pump activity through generation of singlet oxygen in the mesentery.

6. Tumor cells-derived chemical substance(s) in the lymphatic system

During cancer progression, metastatic spreading of malignant tumor cells occurs through the lymph and blood vessels; however, the manner by which tumor cells enter the lymphatic system is not clear [Oliver, 2004]. Using lymphatic-specific molecular marker, many groups have shown that tumor cells activate peritumoral and intratumoral lymphangiogenesis [Karpanen, 2001; Skobe et

al., 2001; Stacker et al., 2001; Mandriota et al., 2001; Beasley et al., 2002]. It remains controversial whether metastasizing cancer cells reach the lymph node through intratumoral neovascularized lymph capillaries. Some recent findings indicated that the presence of functional lymph capillaries around tumor is sufficient for lymphatic metastasis and that those tumors do not contain intratumoral lymph capillaries [Padera et al., 2002]. In addition, physiological properties what factors contribute to driving forces for lymph formation through the neovascularized lymphatic capillaries and biorheological properties how the lymphatic capillaries as collapsible tube can have functions in inturatumoral space remain dissolved.

The induction of tumor-mediated lymphangiogenesis by VEGFC promotes breast cancer metastasis [Skobe et al., 2001]. VEGFD induces the formation of intratumoral lymphatic capillaries in a mouse tumor model, and VEGFD expression by tumor cells facilitates the spreading of the tumor to lymph node [Stacker et al., 2001]. VEGFR3 expression is upregulated by the endothelium of blood vessels in breast cancer, and VEGFC secreted by intraductal carcinoma cells acts mainly as an angiogenic growth factor [Valtola et al., 1999]. Increased expression of VEGFC by primary tumors correlates with the dissemination of tumor cells to regional lymph nodes, and VEGFC-induced lymphangiogenesis mediates tumor cell dissemination [Mandriota et al., 2001].

We also demonstrated that basic fibroblast growth factor (bFGF) facilitated lymphangiogenesis of the cultured lymphatic endothelial cells in vitro [Tan, 1998] and that platelet-derived growth factor (PDGF)-BB also induced lymphangiogenesis of the cultured lymphatic endothelial cells and promoted lymphatic metastasis [Cao et al., 2004].

6.1 Melanoma cells-derived substances inhibits lymphatic pump activity

In contrast, physicochemical and pharmacological factors affecting lymphangiogenesis and lymphatic metastasis of carcinoma cells remains still unclear. Therefore we attempted to investigate whether supernatant cultured with malignant (metastatic) and benign (non-metastatic) melanoma cell lines; B16-BL6 and K1735 or the malignant Lewis lung carcinoma cell line (LLC) can regulate lymphatic pump activity by using bioassay preparations isolated from murine iliac lymph vessels [Nakaya et al., 2001].

Figure 9 shows representative tracings of the effects of B16-BL6, transformed 3Y 1, LLC, and K1735 supernatants diluted by 20 % and culture medium itself (vehicle) on the rhythmic pump activity of the bioassay preparation. B16-BL6 and

LLC supernatants caused a significant dilation of the lymph vessel with cessation of the pump activity [Fig. 9A & D]. In contrast, both transformed 3Y 1supernatant and vehicle had no significant effect on lymphatic pump activity [Fig. 9B & C]. In 11 out of 15 bioassay preparations, K1735 supernatant also produced no significant effect on lymphatic pump activity. There was no significant tachyphylaxis in the B16-BL6 supernatant-mediated inhibitory response of the lymphatic pump activity. Pretreatment with 3 x 10^{-5} M L-NAME or 10^{-6} M glibenclamide caused significant reduction of the B16-BL6 supernatant-mediated responses. Simultaneous treatment with 10^{-3} M L-arginine and 3 x 10^{-5} M L-NAME significantly lessened the L-NAME only-induced inhibition of the B16-BL6 supernatant-mediated response, suggesting that endogenous NO plays important roles in the supernatant-mediated inhibitory responses. Chemical treatment dialyzed substances of < 1,000 molecular weight (MW), producing complete reduction of the B16-BL6 supernatant-mediated response. In contrast, pretreatment with heating or digestion with protease had no significant effect on the supernatant-mediated response. These findings suggest that B16-BL6 malignant tumor cells may release non-peptide substance(s) of < 1,000 MW, resulting in significant cessation of lymphatic pump activity via production and release of endogenous NO and activation of K_{ATP} channels. The chemical substance(s) released from tumor cells may produce edema of the tumor tissue via the significant inhibition of lymphatic pump activity. Microenvironmental edema in the tumor tissues may affect redistribution of tumor cells through the regional lymph vessels, which may contribute, in part, to the occurrence of the sentinel lymph node (SLN). However, further investigation will be needed to investigate patho-physiological roles of edema formation in tumor tissues for governing the spread of carcinoma cells.

6.2 Is ATP or adenosine the candidate of the tumor-derived substance(s)?

Another important aspect of the above-mentioned study is what substance(s) can become the candidate for the B16-BL6 supernatant-mediated inhibitory response of lymphatic pump activity. Recently we demonstrated that ATP or adenosine caused a significant inhibition of the lymphatic pump activity via an involvement of endogenous NO and K_{ATP} channels [Kousai et al., 2004]. Similar to the B16-BL6 supernatant, ATP caused significant dilation with cessation of lymphatic pump activity. Thus, Figure 10A shows representative tracings of the effect of ATP (3 x 10^{-8} ~ 10^{-6} M) on rhythmic pump activity of the isolated rat iliac lymph vessel. Figure 10B & C demonstrate such summarized data of the ATP-mediated changes in % frequency

and cessation period of the lymphatic pump activity, respectively. ATP also significantly increased the maximum (Dmax) and minimum (Dmin) diameters in a dose-dependent manner, resulting in dilation of the isolated lymph vessels. Figure 10D & E demonstrate such summarized data of changes in % Dmax and % Dmin of the isolated lymph vessels, respectively. Removal of the endothelium or treatment with L-NAME significantly reduced the ATP-induced inhibitory responses of the lymphatic pump activity, while the reduction was not addressed completely with 10^{-6} M ATP. L-arginine significantly restored the ATP-induced inhibitory responses in the presence of L-NAME. The ATP-induced inhibitory responses in the lymph vessels with intact endothelium were also significantly, but not completely, suppressed by pretreatment with glibenclamide. 8-Cyclopentyl-1, 3-dipropylxanthine (DPCPX, a selective adenosine A1 receptor antagonist), but not suramine (a P_{2x} and P_{2Y} receptor antagonist) or 3,7-dimethyl-1-proparglyxanthine, (DMPX, a selective adenosine A2 receptor antagonist), significantly decreased the ATP-induced inhibitory responses. α, β–Methylene ATP (a selective P_{2x} and P_{2Y} receptor agonist) produced no significant effect on the lymphatic pump activity. In some lymph vessels with intact endothelium (24 out of 30 preparations), adenosine also caused dose-dependent dilation with cessation of the lymphatic pump activity. L-NAME reduced significantly the lower ($3 \times 10^{-8} \sim 3 \times 10^{-7}$ M) concentrations of adenosine-induced inhibitory responses. Glibenclamide or DPCPX also suppressed significantly the adenosine-induced inhibitory responses. We concluded that ATP dilates and inhibits rhythmic pump activity in the isolated iliac lymph vessels in endothelium-dependent and –independent manners. The ATP-mediated inhibitory responses may be, in part, related to production of endogenous NO, involvement of K_{ATP} channels or activation of adenosine A1 receptors in the lymphatic smooth muscle and endothelium.

6.3 PTHr-P-mediated generation of NO and activation of K_{ATP}

Parathyroid hormone-related peptide (PTHr-P) was originally found as a tumor-derived humoral factor [Philbrick et al., 1996; Winquist et al., 1987]. PTHr-P is also known to affect the cardiovascular system as an autocrine and/or paracrine factor [Schlüter and Piper, 1998]. Thus PTHr-P reduces systemic blood pressure in vivo and causes relaxation of vascular smooth muscles [Maeda et al., 1999; Qian et al., 1999; Sutliff et al., 1999; Takahashi et al., 1995]. It may be reasonable to hypothesize that, as it is a macromolecular substance, the tumor cells-derived PTHr-P can easily penetrate lymphatic capillaries and then inhibit the rhythmic pump

activity of collecting lymph vessels. This may contribute to formation of edema in tumor tissues, increasing of hydrostatic pressure in tissue space, and dilution of tumor cells-derived substances including cytokines, growth factors, and active substances on lymph vessels such as PTHr-P. Dilution of PTHr-P and the other substances [Ohhashi, 1993] that stimulate rhythmic pump activity of the collecting lymph vessels may facilitate lymphatic spread of carcinoma cells by allowing lymph clearance from tumor sites. Increased lymph clearance may provide routes of tumor cells to reach the primary lymph node.

Thus we attempted to investigate the effects of PTHr-P (1-34), a PTH receptor-binding domain, on the rhythmic pump activity in isolated pressurized iliac lymph vessels of mice. Low concentrations (1×10^{-10} and 3×10^{-10} M) of PTHr-P (1-34) dilated the lymph vessels and reduced the frequency of pump activity; whereas high concentrations (1×10^{-9} to 1×10^{-8} M) of the PTHr-P caused dilation with cessation of the lymphatic pump activity. L-NAME (3×10^{-5} M), but not indomethacin (10^{-5} M), significantly reduced the PTHr-P-mediated inhibitory responses of the lymphatic pump activity. In the presence of L-NAME and L-arginine (10^{-3} M), the L-NAME-mediated inhibition of the PTHr-P-mediated responses was significantly reduced. Glibenclamide (1×10^{-6} M) significantly suppressed the inhibitory responses of the lymphatic pump activity produced by the PTHr-P or S-nitroso-N-acetyl-penicillamine. The PTHr-P-mediated inhibitory responses were significantly reduced by treatment with PTHr-P (7-34, 10^{-7} M). These findings suggest that PTHr-P (1-34) inhibits rhythmic pump activity of the isolated murine lymph vessels via PTH receptors and that production and release of endogenous NO and activation of K_{ATP} channels in the lymph vessels contribute to the PTHr-P-mediated inhibitory responses.

7. Conclusions and future perspectives

In many species including human beings and many organs including gastrointestinal tracts, the collecting lymph vessels demonstrate clearly rhythmic spontaneous contractions, which play crucial roles in lymph transport through the lymphatic system in vivo and then seem to affect lymph formation via changing pacemaker sites of the spontaneous contractions and contractile pattern of lymphangions. The rhythmic pump activity also works physiologically under the specific environment with lower oxygen tension in lymph (20 ~ 35 mmHg). In addition, generation of endogenous NO and/or activation of K_{ATP} channels play pivotal roles in the regulation of the lymphatic pump activity. The ROS may become one

of key substances that affect significantly the lymphatic pump activity in patho-physiological conditions such as inflammation, metastasis of carcinoma cells, and reperfusion injury. Chemical substances are released from malignant melanoma cells or lung carcinoma cells, the candidate of which seems to be ATP or adenosine. The tumor-derived chemical substances and PTHr-P caused a significant dilation of lymph vessels with cessation of the lymphatic pump activity through generation of endogenous NO and activation of K_{ATP} channels, resulting in formation of edema within tumor tissues. Thus we have concluded that three key factors in the lymphatic system may be 1) lower oxygen tension in lymph, 2) generation of endogenous NO in the endothelial cells, and 3) activation of K_{ATP} channels in lymph vessels. Thus we should be taken accounts into these key factors to evaluate the regulation of lymph formation, lymph transport, and functions of lymph nodes in physiological and patho-physiological conditions. All of the three key factors inhibit lymph transport, resulting in formation of edema. Thus it can be speculated that the key factors work non-selectively and rapidly to dilute the toxic and irritated substances released from devastating pathogens or tumor cells. Further investigations will be needed to find the excitatory substances of lymph formation and/or lymph transport that may work against the formation of edema in the tissues.

However, important questions remain unanswered; how can the three key factors affect on tumor-mediated lymphangiogenesis and lymphatic metastasis of carcinoma cells? How can the three factors affect on the development and determinant of sentinel lymph nodes? How can the three factors interact with the controlling factors in the interstitial space?

Finally we would like to propose our hypothesis regarding another important aspect of the lymphatic system. The lymphatic system is concerned with the immune system. Lymph nodes are the organs where innate immune responses lead to acquired immunity, where some of our most devastating pathogens evade immunity, and where auto-reactive lymphocyte first encounter tissue-specific self-antigens and are either tolerized or activated. The main role of the lymphatic system is concerned with the return of plasma proteins from tissue interstitium to vascular compartment. This need arises from the uni-directional movement of proteins across the venular endothelium. Tissues require perfusion with plasma proteins have been considered as to supply nutritional elements as well as remove waste products. Recently we have confirmed that the lymphatic movement of plasma proteins and the concentrating mechanisms of the proteins through

small-sized lymph vessels seem to contribute specifically to the innate immunity. The regulation of innate immunity may be related to the concentration of plasma proteins-mediated release of lymphocytes including natural killer cells from regional lymph nodes into the efferent lymph vessels. The important roles of plasma protein through the lymphatic system will be also evaluated in the future with special reference to dynamic properties of lymphocytes in the lymphatic system.

Our hypothetical scheme explaining the lymphatic system with physiology and pharmacology is shown in Figure 11.

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Legends

Figure 1. Passive pressure-radius (○), total pressure-radius (●), actively generated pressure-radius (△) relationships in single lymphangion specimens isolated from bovine mesenteric collecting lymph vessels. Vertical bars stand for standard errors. Data from Ohhashi et al. (1980), by permission.

Figure 2. Representative responses of two kinds of pumping preparations in bovine mesenteric collecting lymph vessels to electrical stimulations, which are rectangular pulses of 50 V, 0.5 ms in duration at 2 Hz. The pacemaker sites are situated in valvular (right panel) and intervalvular (left panel) region, respectively. P=pacemaker position of the rhythmic spontaneous contractions in each preparation; P'=new pacemaker position of the contractions; IS=recording position of outer diameter of the lymph vessels in each preparation; E=electrical stimulating position in each preparation. Data from Ohhashi (1987), by permission.

Figure 3. A: Representative tracings of the effects of Y 27632 (1,3, and 6 μM) on the rhythmic pump activity of the lymph vessel. The arrows indicate the starting and ending points, respectively, for the extraluminal superfusion of Y 27632 through the lymph vessels. Effects of Y 27632 on the percent maximum diameter (%Dmax, B), percent minimum diameter (%Dmin, C), % frequency (D), and the period of cessation (E) of the lymphatic pump activity (n=8). *P<0.05, significant difference from the 1 μM Y 27632-induced responses. Data from Hosaka et al. (2002), by permission.

Figure 4. Representative tracings of changes in systemic arterial pressure (upper panel), the flow rate of lymph (middle panel), and PO_2 in lymph (lower panel) in an anesthetized dog before and after an intravenous administration of 3 M KCl solution. The marker is a time-scale of 2 min. Data from Ohhashi et al. (2003, in Japanese), by permission.

Figure 5. Transverse section of blood capillary found within the external longitudinal smooth layer of bovine mesenteric collecting lymph vessels. The endothelium of the blood capillary has many pedicles that protrude into the lumen. Continuous basement membrane is found around the blood capillary. X 2400. Data from Ohhashi et al. (1977), by permission.

Figure 6. Immunolocalization of ecNOS and iNOS in the cultured lymphatic endothelial cells of canine thoracic ducts. In the panel A, the cells incubated with antibody ecNOS show the staining reaction in the nuclei and the cytoplasm. In the panel B, the cells incubated with anti-iNOS show the reaction in the cytoplasm. The marker is 100 μM. Data from Nojiri and Ohhashi (1999), by permission.

Figure 7. Representative pseudo-color images of [Ca^{2+}]-transient (low concentration; blue, yellow, and red; high concentration) in the cultured lymphatic endothelial cell of canine thoracic duct before (A) and 40 s (B) after an administration of 10^{-5} M histamine into the culture medium. X 1000. Data from Ohhashi et al. (2003, in Japanese), by permission.

Figure 8. Representative photographs of a phase-contrast image (A) and of the arrangement of F-actin (B) in rat cultured lymphatic endothelial cells. The markers in the (A) and (B) show 100 and 50 μm, respectively. Data from Mizuno et al. (2003), by permission.

Figure 9. Representative tracings of the B16-BL6 supernatant (A)-, vehicle (B)-, transformed 3Y1 supernatant (C)-, Lewis lung carcinoma (LLC) supernatant (D)-, and K1735 supernatant (E)-induced responses of rhythmic pump activity in murine isolated iliac lymph vessel. Thick black bars show the period of superfusion of the supernatant or vehicle. Data from Nakaya et al. (2001), by permission.

Figure 10. Representative tracings of dose-dependent effects of ATP (3 x 10^{-8} M – 10^{-6} M) on the rhythmic pump activity in a rat isolated iliac lymph vessel (A). The up-arrow heads and down-arrow heads indicate starting and ending points of the administration of the agents, respectively. Reproducible effects of ATP (the first; open circle. the 2nd; closed triangles, the 3rd; closed squares) are obtained on the % frequency and cessation period (C) of the lymphatic pump activity, and % Dmax (D) and % Dmin (E) of the isolated lymph vessels with intact endothelium (n=6). The % frequency is calculated by relative extent of changes in the frequency (times/min) of lymphatic pump activity after to before the application of the agent. The cessation period (sec) indicates the maximum cessation period of lymphatic pump activity induced by the agent. Data from Kousai et al. (2004), by permission.

Figure 11. Our hypothetical scheme explaining the lymphatic system.

Table 1. Different and common properties between cultured arterial endothelial cells (ECs) and lymphatic ECs (rat).

properties	Arterial ECs	Lymphatic Ecs (rat)
cobblestone appearance	Yes	Yes
presence of F-actin	Yes	Yes
phagocytosis of Dil-Ac-LDL	Yes	Yes
presence of Factor VIII-related antigen	Yes	Yes
sensitivity to PO_2 in culture	Low	High
Immunoreactivity of ecNOS	Low	High
expression of LYVE-1	No	Yes
expression of Prox-1	No	Yes
expression of podoplanin	No	Yes
expression of VEGFR-3	No	Yes