

AN AVIAN MODEL FOR STUDIES OF EMBRYONIC LYMPHANGIOGENESIS*

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ABSTRACT

Embryonic development of lymphatics (lymphangiogenesis) in recent years has rarely been studied experimentally. Using an avian model, we showed that both intra- and extra-embryonic blood vessels of chick and quail embryos are accompanied by lymphatics. The lymphatics of the chorioallantoic membrane (CAM) are drained by lymphatic trunks of the umbilicus and are connected to the posterior lymph hearts. Intra-embryonic lymphatics are drained via paired thoracic ducts into the jugulo-subclavian junction. The lymphatic endothelial cells are characterized by the expression of Vascular Endothelial Growth Factor Receptors (VEGFR) -2 and -3. Application of VEGF-C, the ligand of these two receptors, on the differentiated CAM, induces proliferation of lymphatic endothelial cells and formation of huge lymphatic sinuses. These lymphatics derive from pre-existing lymphatic endothelial cells, whereas, in early embryos lymphangioblasts are present in the mesenchyme. This phenomenon can be demonstrated by interspecific grafting experiments between chick and quail embryos. Together with the early lymph sacs, the lymphangioblasts form the embryonic

lymphatic system. Our studies demonstrate the importance of lymphangioblasts and lymphangiogenic growth factors in embryonic lymphangiogenesis.

About 8-9 decades ago the development of embryonic lymphatics was studied intensively. Since then our knowledge in this field has not appreciably increased, and it is still unknown whether lymphatics derive by sprouting from veins, de novo from lymphangioblasts, or by both mechanisms. Recent studies, however, have shown that the Vascular Endothelial Growth Factor-C (VEGF-C) is a highly specific lymphangiogenic growth factor (1,2). This observation raises the possibility of studying new questions and perspectives.

Compared to the studies on the development of blood vessels, studies on the development of lymphatics are rare. This dichotomy has led in the last decades to use of the term "angiogenesis" to define the process of blood vessel development, although the expression "hemangiogenesis" is probably more appropriate. This terminology, however, interferes with the expression "hemangioblast," which is generally used to define a precursor cell that gives rise to both blood cells and blood vessel endothelium. Perhaps the term "hemendothelioblast" would be more accurate. In this paper, we will use the term "angiogenesis" for the formation of blood

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vessels, and “lymphangiogenesis” to include the mechanisms involved in the development of lymphatics (reviews see: 3-5).

Like developing blood vessels, the first anlagen of lymphatics are solely made up of endothelial cells. Development of lymphatics has been studied by injection methods, serial sections (6-8), and in living animals (9,10). Nonetheless, given the lack of specific markers, the origin of lymphatic endothelial cells remains unknown. Thus, are they derived from lymphangioblasts of the early mesenchyme (11,12), or from veins by sprouting (6,13), or by both mechanisms (14)? Blood vascular endothelial cells clearly derive by multiple mechanisms; from angioblasts and hemangioblasts, and also from growth of early embryonic vessels (review see: 15). If the assumption holds true that lymphatic endothelial cells are derived from veins, and grow exclusively by sprouting, then the principal difference between angiogenesis and lymphangiogenesis would reside in the absence of a lymphangioblastic cell lineage. This concept appears to be supported by the fact that lymphatics develop much later than blood vessels. In the human, lymph sacs have been found in 6-to 7-week-old embryos of 10-14 mm total length (8). This timing is about 3 to 4 weeks after the development of the first blood vessels. In the chick, the deep lymphatic system is first detectable during day 4-5 of incubation (16), whereas the first blood vessels are already seen after 1 day of incubation (17). It has been assumed, however, that besides the venous origin of lymphatics there are endothelial lined clefts that become integrated into the growing lymphatic system (12,18,19).

Despite the uncertainty about the existence of multiple lymphatic anlagen, it is generally accepted that much of the lymphatic system is derived from endothelial lined lymph sacs, which are located immediately adjacent to veins. Studies on mammals have shown that there are eight lymph sacs; three paired and two unpaired (6,20). The paired anlagen are the jugular, subclavian and

posterior lymph sacs, and the unpaired are the cisterna chyli and the retroperitoneal (mesenteric) lymph sac. In the human, the subclavian lymph sac is an extension of the jugular lymph sac (6,8). Except for the cisterna chyli, the lymph sacs become transformed primarily into lymph nodes. The lymph sacs are derived by fusion of lymphatic capillaries filled with stagnant blood, suggesting an origin from veins (21,22). The blood is removed into the veins when the lymphatics begin to function (9).

We studied the development of the lymphatics in chick and quail embryos. Descriptive studies were performed with histological and immunohistochemical methods, and in situ hybridization with VEGFR-2 and -3 probes. Two kinds of experiments were then carried out: 1. Wing buds of early chick embryos were grafted homotopically into quail embryos, to study whether the limb bud mesoderm already contained lymphangioblasts, or, whether in contrast, the wing lymphatics were exclusively derived from sprouts of the jugulo-axillary lymph sacs. 2. The growth factor VEGF-C was applied on the differentiated chorioallantoic membrane (CAM) of 13 day-old chick embryos to study its lymphangiogenic potency. Our experiments showed that VEGF-C was a highly specific lymphangiogenic growth factor for VEGFR-2- and -R3-positive lymphatic endothelial cells. Our grafting experiments demonstrated that there were lymphangioblasts in the early avian mesenchyme. Therefore, embryonic lymphatics did not grow exclusively by sprouts of the early lymph sacs, but also by recruitment of local lymphangioblasts.

MATERIAL AND METHODS

Embryos

Fertilized chick and quail eggs were incubated in a humidified atmosphere at 37.8°C. On various incubation days, normal and experimental embryos were fixed

overnight. They were dehydrated and embedded in paraffin wax for immunohistochemistry and in situ hybridization studies, or in Epon resin for semi- and ultra-thin sectioning.

Hybridization of paraffin sections

Normal and experimental embryos were fixed overnight at 4°C in Serra's fixative (23). The samples were dehydrated, embedded in paraffin wax and 8µm sections mounted onto silanized slides. The sections were post-fixed in 4% paraformaldehyde solution (PFA) and, in older specimens, treated with proteinase K and refixed in 4% PFA. The quail VEGFR-3/*Quek2* and VEGFR-2/*Quek1* mRNA riboprobes was a generous gift from Dr. Anne Eichmann (Nogent-sur-Marne). *Quek2* has 70% identity with the human *flt4* (VEGFR-3) gene (24). The 1500 bp probe was cloned into pcDNA/Amp (Invitrogen, San Diego). Linearization was performed with *HindIII* and *XhoI*. The 3kb VEGFR-2/*Quek1* probe was linearized with *HindIII* and *SphI*. The sense and antisense riboprobes (24-26) were labeled as described before with digoxigenin RNA labeling kit as recommended (Boehringer, Mannheim, Germany). A hybridization mixture of 40% formamide, 25% 20X SSC, 1% Denhardt's solution, 1% tRNA, 1% herring sperm DNA, 2% labeled sense or antisense probe and 30% DEPC-treated water was prepared. Sixty µl of hybridization mixture was placed on each slide and the slides were incubated overnight at 65°C. After standard washing, the location of the digoxigenin was detected using a 1:4000 solution of an alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer) in a blocking agent (1% bovine serum albumin in malate buffer) at 4°C overnight. The antibody was detected using BCIP/NBT (Boehringer) in alkaline phosphatase buffer for 3-5 days, revealing a blue reaction product. The background was stained with nuclear fast red and the slides mounted. Representative photographs of the

hybridization sites were photographed onto Kodak Ectachrome and Agfa Ortho film. The VEGFR-3 sense controls did not reveal a specific signal (26).

Immunohistochemistry

Endothelial cells of quail embryos were stained with the QH1 antibody (17; Developmental Studies Hybridoma Bank, Iowa City, IA). Staining was performed according to the indirect peroxidase method as described previously (27). In order to stain all quail cells, we used the anti-quail antibody QCPN (DSHB). The antibody was diluted 1:500. The secondary antibody was peroxidase-conjugated goat-anti-mouse Ig (Sigma, Deisenhofen, Germany), diluted 1:300. DAB was used as chromogen. For staining of the media of vessels, an anti-smooth muscle α -actin (α SMA) antibody (Sigma) was used, diluted 1:5000. Secondary antibody and chromogen were the same as described above.

*VEGFR-3/*Quek2* and QH1 double staining*

In several specimens we combined VEGFR-3 in situ hybridization and QH1 immunofluorescence. For this purpose, in situ hybridization on paraffin sections was performed as described above, with slight modifications. The sections were not treated with proteinase K. Furthermore, the anti-digoxigenin/AP antibody and the QH1 antibody were applied simultaneously; diluted 1:4000 and 1:2000, respectively. Thereafter, the alkaline phosphatase reaction was performed as usual. When the blue reaction product in the lymphatics was visible in the sections, the alkaline phosphatase reaction was stopped with 1% EDTA in alkaline phosphatase buffer. The slides were rinsed with A. dest. and PBS and the sections were blocked with 1% BSA, and again incubated with QH1 antibody, 1:2000. After several washings with PBS, the secondary Cy3-conjugated goat-anti-mouse antibody (Dianova, Hamburg, Germany) was applied;

diluted 1:200. The slides were rinsed in PBS and mounted with Moviol (Hoechst, Frankfurt a. M., Germany). The sections were studied with Zeiss Axioskop using brightfield and fluorescence illumination.

Grafting experiments

In order to study the origin of the lymphatics in the wing, the distal part of the wing bud of 3 - 3.5 day-old quail embryos was replaced by a corresponding wing bud of a HH stage 21-22 chick embryo (28), as described previously (29). The quail embryos were reincubated until day 10. The chimeric wings were fixed in Serra's solution, embedded in paraffin and sectioned serially at 8 μ m. The sections were studied with the QH1, QCPN and α SMA antibodies, with VEGFR-3/*Quek2* in situ hybridization, and with *Quek2* and QH1 double staining.

Ultrastructure

Specimens were fixed in 3% glutaraldehyde and 2% formaldehyde in 0.12 M sodium cacodylate buffer, postfixed with 1% osmium solution, immersed with uranyl acetate and embedded in Epon resin (Serva, Heidelberg, Germany). Semithin (0.75 μ m) and ultrathin (70 nm) sections were cut with an Ultracut S (Leika, Bensheim, Germany). Semithin sections were stained with AzurB/Nileblue. Ultrathin sections were studied with an EM 10 (Zeiss, Stuttgart, Germany).

Proliferation studies

Proliferation of cells was monitored with the BrdU/anti-BrdU method (30) after application of VEGF-C on CAM of 13-day-old chick embryos. One or two days later, the embryos were incubated with 100 μ l of a 40mM BrdU solution (Sigma) for 45 min. The specimens were then fixed with ethanol containing 3% acetic acid. Paraffin sections were stained as previously described (31).

CAM-assay

Fertilized eggs of the White Leghorn chick (*Gallus gallus*) were incubated at 37.8°C and 80% humidity. On day 4 of development, a window was made into the egg shell. The embryos were checked for normal development and the eggs sealed with cellotape. They were further incubated until day 13. Thermanox coverslips (Nunc, Naperville, IL) were cut into discs of about 5 mm in diameter. Sterile and salt-free growth factors (VEGF-A, VEGF-C) were dissolved in distilled water and about 3.3 μ g/5 μ l were pipetted on the discs. After air-drying, the inverted discs were applied on CAM. After three days, the specimens were fixed in 3% glutaraldehyde and 2% formaldehyde and rinsed in 0.12 M sodium cacodylate buffer. They were photographed with a stereomicroscope (Wild M8) and embedded for semi- and ultrathin sectioning according to standard procedures. Controls were performed with a great number of different proteins.

Injection method

The lymphatics of 10 to 16 day-old chick and quail embryos were perfused with glutaraldehyde/formaldehyde fixative. Two milliliters of Mercox-blue (Norwald, Hamburg, Germany) were mixed with 25 μ l of accelerator and injected into the lymphatics of the CAM or the umbilicus using fine glass needles and a micromanipulator.

RESULTS

Lymphatics of chick embryos were documented by injecting tracer (Mercox-blue) into the umbilical lymphatic trunks (2,32). By this means, the deep and superficial parts of the posterior lymph sacs of day 10 chick embryos can be demonstrated. In day 16 chick and day 14 quail embryos, it is possible to demonstrate the lymphatics of the trunk. From the umbilical region the tracer flows

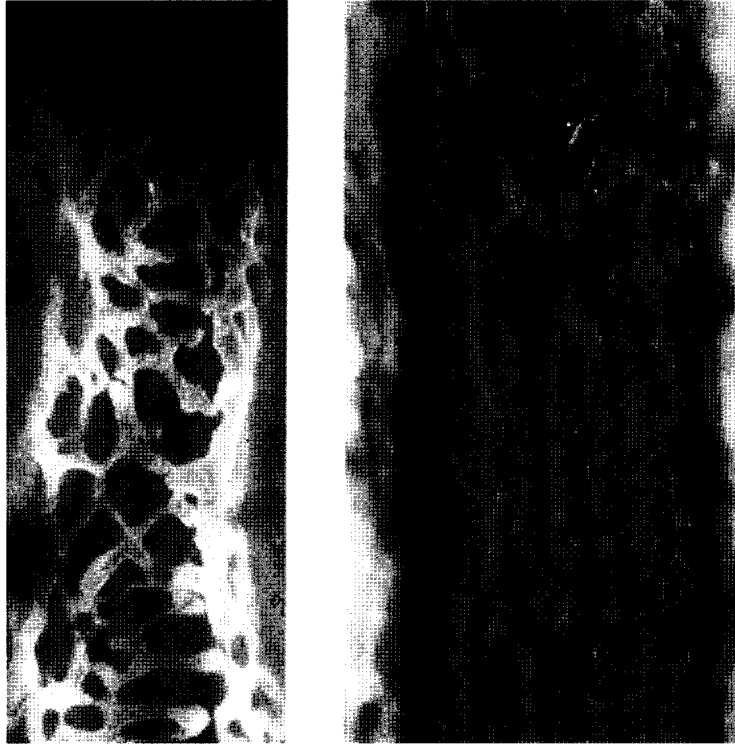


Fig. 1. Intra-lymphatic injection of Mercox-blue. Left — Illustration of the lymphatic capillaries surrounding a vein of the CAM of a day 16 chick embryo. X20; Right — Illustration of the paired thoracic duct (D) and the lymphatic plexus surrounding the abdominal aorta of a day 14 quail embryo. X15.

into both directions towards the embryo and into the chorioallantoic membrane (CAM), indicating that there are no or only a small number of valves within the lymphatics. In the CAM both the arterial and venous vessels are accompanied by lymphatics. Arteries and arterioles are accompanied by a pair of lymphatics and a dense plexus of lymphatics is located around the large veins (*Fig. 1, left*). The intraembryonic, abdominal and thoracic lymphatics can be injected up to the point where the paired thoracic duct reaches the paired cranial caval vein. Dense lymphatic plexuses can be observed around both arteries and veins. Numerous lymphatics surround the abdominal aorta (*Fig. 1, right*) and its caudal extension, the median sacral artery. The paired thoracic duct is the continuation of the lymphatics of the abdominal aorta (*Fig. 1, right*). Before reaching the cranial

caval vein, an anastomosis between right and left thoracic duct can be observed. The arterial branches emerging from the abdominal aorta are also accompanied by lymphatics. Among these, the external iliac artery supplies the upper thigh, whereas the ischial artery is the main vessel of the leg. The injection medium also fills the lymphatics that accompany the branches of the thoracic aorta, the celiac and the cranial mesenteric artery. The cranial mesenteric artery supplies the small intestine and the yolk sac. A very dense plexus of lymphatics accompanies this artery and can be traced into the yolk sac. The cranial mesenteric vein is located beside the artery and is also ensheathed by lymphatics. The caudal mesenteric vein which drains the cloacal region and the rectum is also surrounded by numerous lymphatics. The lymph hearts,

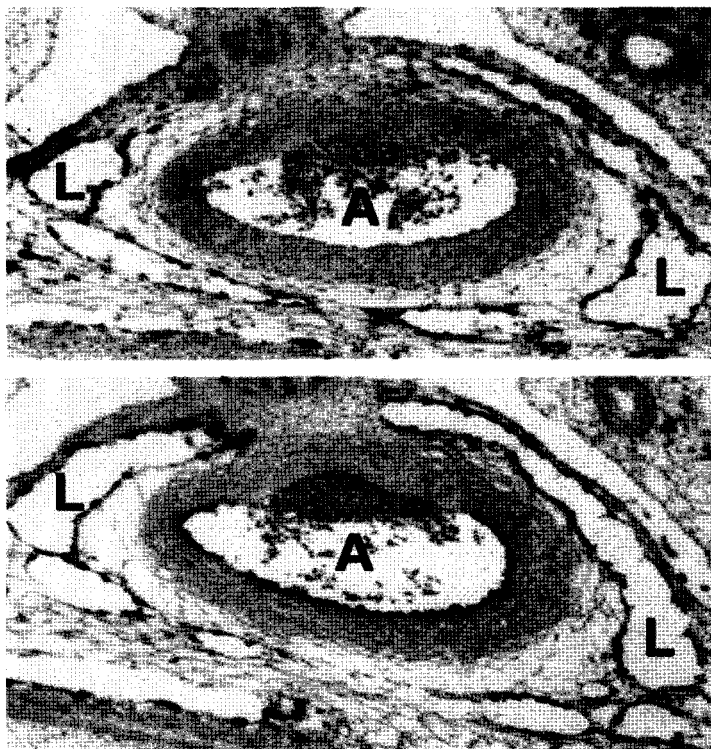


Fig. 2. *In situ* hybridization of a paraffin section of a day 10 quail embryo with a VEGFR-3 probe. Upper—The endothelium of the aorta (A) is negative, accompanying lymphatics (L) are positive. X30; Lower—The endothelium of both the aorta (A) and the lymphatics (L) is positive. X30.

originally observed in the chick by Budge (33) and Sala (34), are located in the dermis of the sacrococcygeal transition, and derive from the superficial part of the posterior lymph sacs.

The lymphatic and the blood vascular endothelium of quail embryos can be stained with the QH1 antibody (17). This antibody does not differentiate between the two types of endothelial cells, but it is specific for the quail and does not stain any chick cells. However, a distinction between blood vessels and lymphatics can be made by *in situ* hybridization with VEGF receptor probes. In the differentiating tissues of avian embryos, VEGFR-3 is a highly specific marker of lymphatic endothelial cells (*Fig. 2, upper*). In early embryos, however, VEGFR-3 is expressed in both blood and lymph vessels, but it is switched off in blood vessels during tissue maturation. Therefore, in day 10 quail

embryos VEGFR-3 is a marker of the lymphatics of the body wall, the limbs and most internal organs. In the lung, where maturation takes place very late, there is prolonged expression of VEGFR-3 in the blood vessels. The same expression pattern can be observed during maturation of the extraembryonic tissues, such as the CAM. In contrast, VEGFR-2 is expressed in both the endothelium of arteries and veins and of lymphatics of day 10 quail embryos (*Fig. 2, lower*). Application of VEGF-A (which binds to the VEGF receptors -1 and -2) on the CAM of day 13 chick embryos induces the expression of VEGFR-2 but not R-3 in the blood vascular capillaries. This growth factor induces angiogenesis but not lymphangiogenesis in the CAM (data not shown). The combination of the VEGF receptors -2 and -3 is characteristic of the

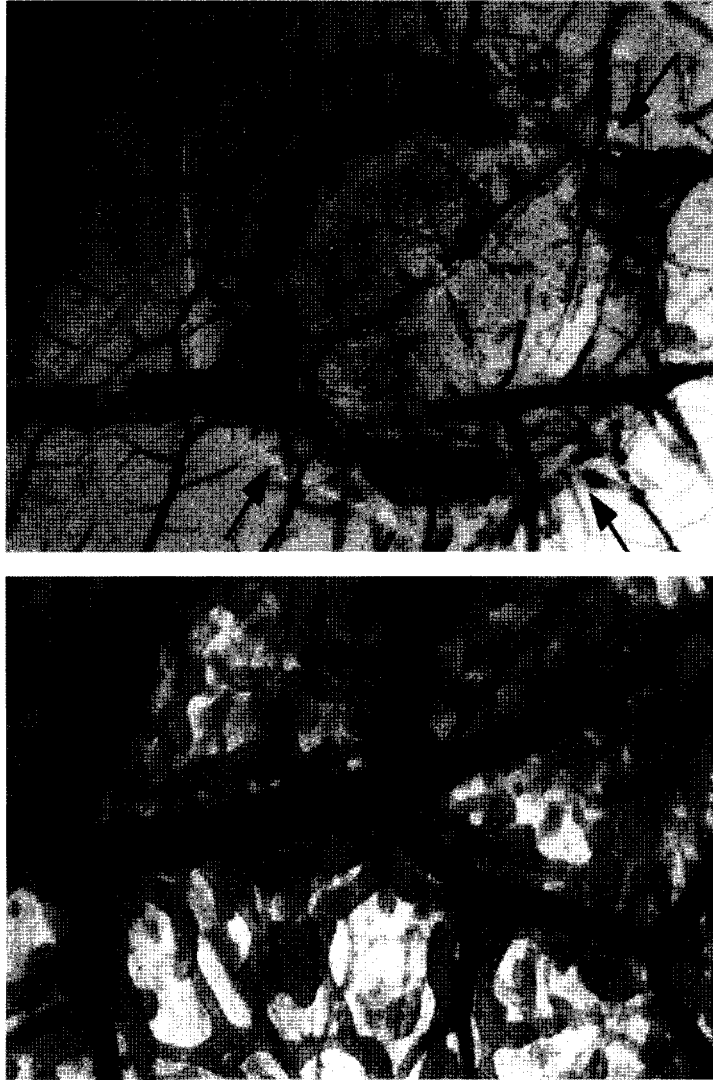


Fig. 3. Application of VEGF-C on the differentiated CAM of day 13 chick embryos. After 3 days the specimens were fixed and intra-lymphatic injection of Mercox-blue was performed. Upper — Note the circular lymphatic sinus (arrows) in the application area. X5; Lower — Higher magnification of the specimen in Fig. 5 showing the high density of lymphatics in the application area. X30.

lymphatic endothelial cells. The ligand of these two receptors is VEGF-C. Application of VEGF-C on the CAM of day 13 chick embryos induces lymphangiogenesis (Figs. 3-5), and only a mild angiogenic side effect can be observed. Injection of Mercox-blue into the lymphatics shows that the newly developed lymphatic sinuses are found only

in the circular application area (Fig. 3). The circular distribution of the growth factor is due to the method of drying of the protein solution on the carrier disc. The histological studies reveal the thin endothelial lining of the lymphatics induced by VEGF-C (Fig. 4). Proliferation studies with the BrdU/anti-BrdU method show that in the differentiated

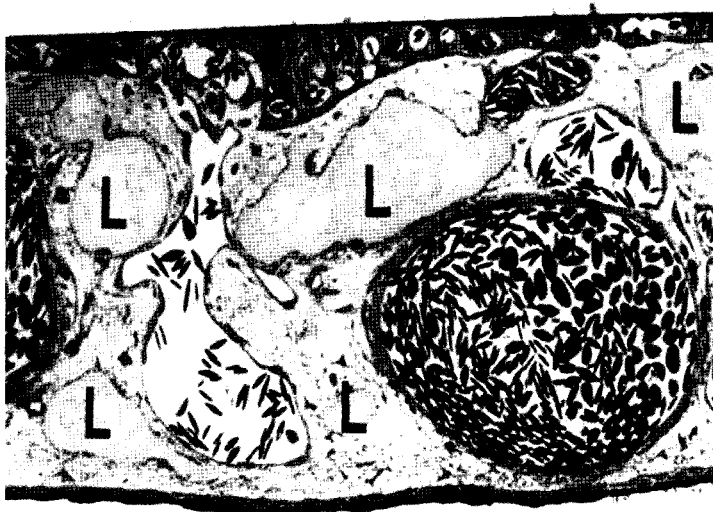


Fig. 4. Semi-thin section of the specimen shown in Fig. 3. Note the numerous lymphatic capillaries (L) which are filled with Mercox-blue. X130.

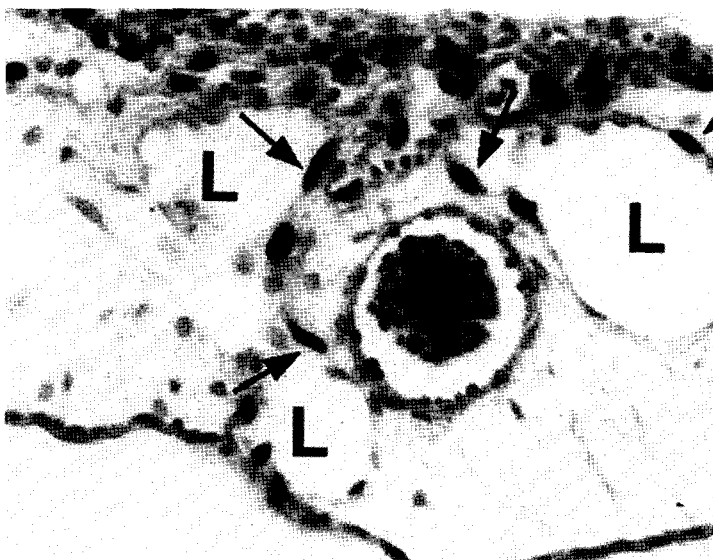


Fig. 5 . Proliferation studies with the BrdU/anti-BrdU method two days after application of VEGF-C on the CAM of day 13 chick embryos. Note the numerous BrdU-positive nuclei (arrows) of the lymphatic capillaries (L). X130.

CAM of control embryos, labeling of lymphatics is an extremely rare event. However, after application of VEGF-C, a high labeling index of lymphatic endothelial cells is found (Fig. 5). In the differentiated CAM, VEGF-C induces proliferation and growth of pre-existing lymphatics.

In early embryos, the first histologically detectable anlagen of the lymphatics are the so-called lymph sacs (Fig. 6). In order to determine the origin and growth of the lymphatics in early embryos, we replaced distal wing buds of 3.5 day-old quail embryos by corresponding wing buds of chick

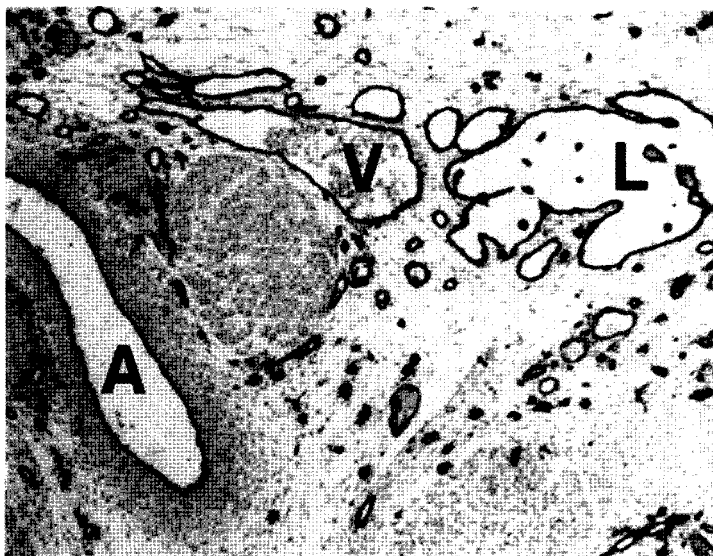


Fig. 6. Staining of the endothelial cells of a day 6.5 quail embryo with the QH1 antibody illustrating the aortic arch (A), the jugular vein (V) and the jugular lymph sac (L). X30.

embryos, as described recently (29). The experiment was performed to determine whether the lymphatics of the wing are exclusively derived by sprouts of the jugulo-axillary lymph sacs, or whether lymphangioblasts are present in the early wing bud. The embryos were reincubated until day 10. In 25 out of 80 experiments, chimeric wings with grossly normal morphology could be harvested. Serial sections of these wings were further analyzed and showed that the vascular pattern was generally normal in all specimens. Arteries and veins could be identified by their localization and the differences in the thickness of the media (data not shown). The chimeric nature of the experimental wings was studied with the QCPN antibody, which stains the nucleus of quail cells. As could be expected, the nerves, which are derived from the neural tube and the neural crest, were of quail origin, whereas most connective tissue cells were of chick origin (data not shown). The origin of the vascular endothelium was studied with the QH1 antibody. These studies showed that the vessels in the distal wing (autopodium and

zeugopodium) were mostly of chick origin, but quail endothelial cells were regularly integrated into the endothelial lining of arteries, veins and microvessels. We also performed VEGFR-3/*Quek2* and QH1 double staining of serial sections of the chimeric wings. With this method it was possible to identify both the lymphatics by their VEGFR-3 expression and, at the same time, the origin of the endothelial cells by the QH1 signal. The results showed that the lymphatics in the distal parts of the chimeric wings were made up of both chick and quail endothelial cells (Fig. 7). Similar to the normal wings, the lymphatics accompanied the main blood vascular routes such as the deep radial artery and the basilic vein. The chimerism of the endothelium showed that the distal wing buds of day 3.5 chick embryos already contained lymphangioblasts that were integrated into the growing lymphatics of the wing.

DISCUSSION

Congenital hypoplasia of the lymphatics

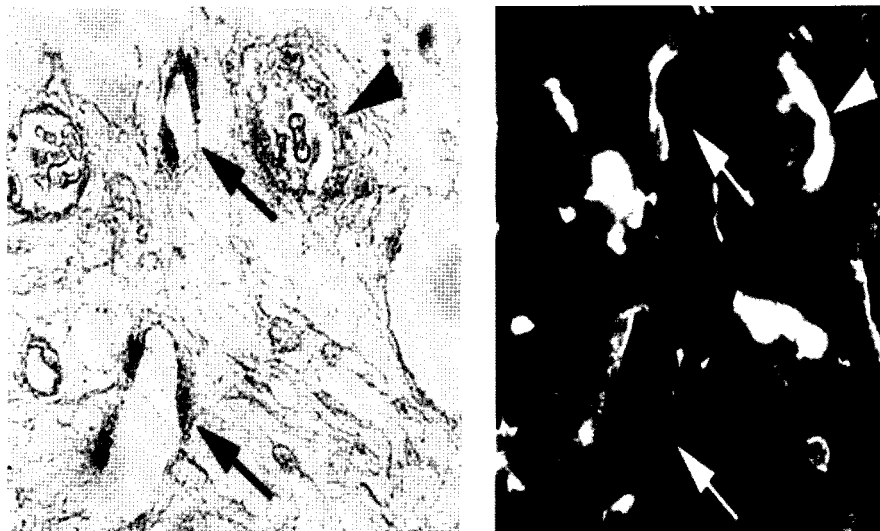


Fig. 7. VEGFR-3 in situ hybridization (left) and QH1 immunofluorescence (right) double staining of a paraffin section of the forewing of a day 10 quail/chick chimera. Note that the endothelium of both the interosseous artery (arrowhead) and the lymphatics (arrows) are chimeric; quail endothelial cells being QH1-positive, chick endothelial cells QH1-negative. X150.

and failure of lymphatics to regenerate result in primary and secondary lymphedema (25,36). Abnormal proliferation of lymphatic endothelial cells may be the key process in lymphangioma, lymphangiosarcoma and Kaposi sarcoma (37). The fact that the lymphatic system has gained less attention probably resides in the methodical problem of detection of the lymphatics. In routine histology, lymphatics are difficult to recognize and to distinguish from blood vessels. The absence of a basal lamina which can be stained in blood vessels with antibodies against type IV collagen and laminin has been used to distinguish between the two types of vessels (38). Antibodies with high specificity for rat and mouse lymphatic endothelium have been produced (39-41). Furthermore, 5' nucleotidase activity of lymphatic endothelium has been observed in histochemical studies (42). Analysis of lymphatics is also possible by radiological lymphangiographical methods *in vivo* (37). Injection of fluorescent dyes has been used in microlymphangiography (1,43), and lymphatics can also be studied by magnetic

resonance imaging and lymphangioscintigraphy (44). However, most markers that have been used to stain blood vascular endothelial cells also stain lymphatic endothelium, and only quantitative but no consistent qualitative differences have been found (35).

In recent years, some endothelial receptor tyrosine kinases involved in growth and differentiation of embryonic blood vessels and lymphatics have been found, including the vascular endothelial growth factor receptor (VEGFR) family. The three VEGF receptors (*flt1*, *KDR*, *flt4* named VEGFR-1, -2 and -3, respectively) are almost exclusively expressed in endothelial cells (25,26,45-47). The ligand, VEGF-A, binds VEGFR-1 (*flt1*) and -2 (*KDR*, *flk1*, *Quek1*) with high affinity and induces angiogenesis (blood vessel development) (for review: see 5). This interaction is accompanied by upregulation of VEGFR-2 in blood vascular endothelial cells, whereas the lymphatics are not affected (48). The latter is an unexpected result because at least one of the ligands, namely VEGFR-2, is expressed in lymphatic endothelium. The expression of

VEGFR-1 has not as yet been studied in the lymphatics, and no probe has yet been cloned for the avian model. However, our own unpublished data indicate that a homologous gene is present in the chick. It can be speculated that VEGF-A (and VEGF-C) exert their effects by binding simultaneously to two receptors. Thus, the combination of VEGFR-1 and R-2 may be specific for the blood vascular endothelium, whereas the combination of VEGFR-2 and R-3 is characteristic of the lymphatic endothelium of differentiated tissues. It can also be assumed that formation of receptor heterodimers is important for the transduction of the signal.

VEGF-C is a secreted, disulfide bonded homodimer which has been cloned from human prostatic carcinoma cells (49). It binds VEGFR-2 and -3 (*flt4*, *Quek2*) with high affinity and induces lymphangiogenesis (5). The human VEGF-C gene comprises over 40 kb genomic DNA and consists of 7 exons. It is located on chromosome 4q34 (50). VEGF-C is synthesized as a pre-pro-protein, and becomes proteolytically processed both intra- and extra-cellularly (review see: 51). VEGF-C is highly expressed in the mesenchyme during embryonic development (52), and is also constitutively expressed in adult tissues. This expression suggests a role in both the development and the maintenance of the lymphatics (51). VEGFR-2 expression has been found in both blood vascular and lymphatic endothelial cells during embryonic development of birds (26,48). Expression of VEGFR-3 has been observed in the endothelium of blood vessels only during early embryonic development. Expression then becomes restricted to lymphatic endothelial cells in later stages of murine and avian development (26,47). Immunohistochemical studies have confirmed that VEGFR-3 is a marker of lymphatics in differentiated tissues (53,54). Our studies show that VEGF-C is a highly specific lymphangiogenic growth factor in the avian CAM. This effect has also been observed in VEGF-C overexpressing

mice (1). The mild angiogenic side effect may be due to the relatively high amount of growth factor used in the CAM-assay. The lymphatic endothelial cells of the CAM are thinner than those of blood vascular capillaries, and they do not possess a continuous basal lamina, which characterizes them as lymphatic capillaries. The lymphatics in the umbilical region of day 16 chick embryos are trunks that are invested by a thin layer of smooth muscle -actin-positive smooth muscle cells (2). VEGF-C is a mitogen of the lymphatic capillaries of the CAM as demonstrated by the BrdU studies. It induces hyperplasia of the preexisting lymphatics, and it may be conjectured that such an effect may be useful in the treatment of patients with lymphatic aplasia and hypoplasia.

In differentiated tissues, lymphatics seem to develop only from pre-existing ones. In contrast, a combination of different mechanisms is observed during the development of embryonic lymphatics. In order to determine the origin of the lymphatics in the wing, we grafted distal wing buds (prospective auto- and zeugopodium) of 3.5 day-old chick embryos homotopically into 3 - 3.5 day-old quail embryos, making use of the quail/chick marker system (55). The quail hosts were reincubated until day 10, and normally developed chimeric wings were further examined. In chick embryos, the jugular lymph sacs develop during days 4-5 (16). Our grafting experiment was performed considerably before the development of the jugulo-axillary lymph sacs, and we therefore were able to exclude that the distal wing buds of 3.5 day-old chick embryos already contained any derivatives of the lymph sacs. According to Sabin (6), all lymphatics of the wing developed from sprouts of the jugulo-axillary lymph sacs. If this assumption was true, the distal chick wings in our experiment would have been invaded in a proximo-distal direction by quail lymphatics. In order to identify the lymphatics in the chimeric wings, we developed a double staining technique consisting of VEGFR-3/*Quek2* *in situ*

hybridization and QH1 immunofluorescence. With this method we are able to stain specifically the VEGFR-3-positive lymphatics, and to determine the origin of the lymphatic endothelium. In the distal parts of the experimental wings, the lymphatics were formed by both chick and quail endothelial cells; most of them of chick origin. These results were obtained for both the deep and the superficial lymphatics of the wing. This outcome tends to refute the pure venous origin theory of Sabin (6), as lymphatics of the wing were not exclusively derived by sprouts of the jugulo-axillary lymph sac. In contrast, lymphatic Anlagen were present in the distal wing buds of early avian embryos. Together with the lymphatics derived by sprouts of the early lymph sacs, the local lymphangioblasts form the embryonic lymphatic system. We suggest that the complexity of mechanisms observed in embryonic angiogenesis can also be found in embryonic lymphangiogenesis (for review see: 3). The main difference seems to reside in the fact that lymphatics develop much later than blood vessels. Furthermore, lymphatic capillaries, which, in contrast to blood vascular capillaries, are not invested by a basal lamina and supporting cells such as pericytes, seem to depend on constitutive expression of maintenance factors such as VEGF-C.

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