Dual origin of avian lymphatics

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Abstract

The earliest signs of the lymphatic vascular system are the lymph sacs, which develop adjacent to specific embryonic veins. It has been suggested that sprouts from the lymph sacs form the complete lymphatic vascular system. We have studied the origin of the jugular lymph sacs (JLS), the dermal lymphatics and the lymph hearts of avian embryos. In day 6.5 embryos, the JLS is an endothelial-lined sinusoidal structure. The lymphatic endothelial cells (LECs) stain (in the quail) positive for QH1 antibody and soybean agglutinin. As early as day 4, the anlagen of the JLS can be recognized by their Prox1 expression. Prox1 is found in the jugular section of the cardinal veins, and in scattered cells located in the dermatomes along the cranio-caudal axis and in the splanchnopleura. In the quail, such cells are positive for Prox1 and QH1. In the jugular region, the veins co-express the angiopoietin receptor Tie2. Quail-chick-chimera studies show that the peripheral parts of the JLS form by integration of cells from the paraxial mesoderm. Intra-venous application of DiI-conjugated acetylated low-density lipoprotein into day 4 embryos suggests a venous origin of the deep parts of the JLS. Superficial lymphatics are directly derived from the dermatomes, as shown by dermatome grafting. The lymph hearts in the lumbo-sacral region develop from a plexus of Prox1-positive lymphatic capillaries. Both LECs and muscle cells of the lymph hearts are of somitic origin. In sum, avian lymphatics are of dual origin. The deep parts of the lymph sacs are derived from adjacent veins, the superficial parts of the JLS and the dermal lymphatics from local lymphangioblasts.
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Introduction

The development of the lymphatic vascular system starts considerably later than the blood vascular system. In the chick, the first blood vessels can be seen after 1 day of incubation (Pardanaud et al., 1987), whereas morphological evidence for lymphatic endothelial cells (LECs) is present around day 5 (Clark and Clark, 1920). In the mouse, blood vessel development starts at embryonic day (ED) 7.5 (Breier et al., 1996); the anlagen of the lymphatic vessels (lymphatics) can be seen in the jugular region at ED 10 (Wigle and Oliver, 1999). In the human, jugular lymph sacs have been found in 6- to 7-week-old embryos of 10–14 mm total length (van der Putte, 1975). This is 3 to 4 weeks after the development of the first blood vessels. The first obvious morphological criteria of the developing lymphatics are the lymph sacs, which are located in close vicinity to deep embryonic veins. Studies on mammalian embryos have shown that there are eight lymph sacs; three paired and two unpaired (Sabin, 1909; Töndury and Kubik, 1972). The paired ones are the jugular, subclavian and posterior lymph sacs, and the unpaired are the *Cisterna chyli* and the retroperitoneal (mesenterial) lymph sac. In the human, the subclavian lymph sac is an extension of the jugular lymph sac (Sabin, 1909; van der Putte, 1975). Except for the *Cisterna chyli*, the lymph sacs develop into primary lymph nodes.

The consecutive development of blood vascular endothelial cells (BECs) and lymphatic endothelial cells (LECs) has led to the hypothesis that LECs are derived from BECs, specifically from neighboring veins (Sabin, 1902), and only very recently

Oliver and Harvey (2002) have supported this hypothesis on the basis of the expression pattern of the homeobox transcription factor Prox1. Prox1-deficient mice die at embryonic day 14.5. They possess a normal blood vascular system, but the development of the lymphatics is arrested at ED 10.5 (Wigle and Oliver, 1999). The first Prox1-positive endothelial cells (ECs) are located in the jugular region in the angle formed by the confluence of the anterior and posterior cardinal veins, and it has been postulated that these venous ECs are the precursors for LECs in the embryo (Oliver and Harvey, 2002). By budding and sprouting, the cells seem to give rise to the lymphatic vascular system. However, scattered Prox1-positive cells have been observed in extra-embryonic parts of chick embryos, e.g., the allantoic mesoderm, and quail-chick grafting of the allantoic bud of ED 3 embryos has shown that there are mesodermal sources for LECs apart from the deep embryonic veins (Papoutsi et al., 2001). In fact, early descriptive studies by Huntington (1914) have suggested that the lymphatic vascular system develops without any contribution from embryonic veins; a hypothesis also supported by Kampmeier (1912). By now, the differentiation potency of early venous ECs has not been tested by means of cell lineage studies, which appears to be the only method to study the lymphangiogenic potency of these cells during normal development. We have employed this method in combination with grafting experiments to study the development of the avian jugular lymph sacs (JLS), the superficial dermal lymphatics and the lymph hearts. Our results provide evidence for a dual origin of the lymphatics from both the veins and scattered mesodermal precursor cells.

Materials and methods

Embryos

Fertilized chick and quail eggs were incubated in a humidified atmosphere at 37.8°C. Staging (HH stages) was performed according to Hamburger and Hamilton (1952). The embryos were used for experimental studies and were also fixed at various stages of development for descriptive studies.

Intra-vascular injections

The lymphatics of 13-day-old chick embryos were perfused with 3% glutaraldehyde and 2% formaldehyde fixative. Two milliliters of Merkox-blue (Norwald, Hamburg, Germany) were mixed with 25 μl of accelerator and injected into the lymphatics of the chorioallantoic membrane (CAM) or the umbilicus using fine glass needles and a micromanipulator, as shown previously (Oh et al., 1997; Papoutsi et al., 2001). About 5 μl DiI-conjugated acetylated low density lipoprotein (DiI-ac-LDL) (Paesel and Lorei, Hanau, Germany) at a concentration of 200 μg/ml was injected into native lymphatics of the CAM of day 15 chick embryos. After 20 min of incubation, the specimens were rinsed with physiological salt solution and studied with a fluorescence stereomicroscope after 2–3 h (Leica, Bensheim, Germany). DiI-ac-LDL (5 μl) was also injected in ovo into the vitelline veins of day 4 quail embryos. The embryos were re-incubated until day 6.5, fixed in 4% paraformaldehyde (PFA), immersed in 5% and 15% sucrose and embedded in tissue freeze medium (Leica). 35 μm serial sections were studied with an epifluorescence microscope (Leica, Axioskop), and were additionally stained with QH1 antibody as described below.

Grafting experiments

(A) In order to study the origin of the jugular lymph sacs (JLS), we performed grafting experiments making use of the quail/chick chimera model (Le Douarin, 1969). Two types of experiments were performed: (1) Paraxial mesoderm was isolated from 2-day-old quail embryos and grafted homotopically into corresponding chick embryos. The implantation site was at the level of somites 8–12; (17 experiments). (2) Lateral plate mesoderm at the level of somites 6–13 was isolated from 2 day-old quail embryos and grafted homotopically into corresponding chick embryos (6 experiments). In both experiments, the chick embryos were re-incubated until day 6.5, to study integration of endothelial cells into the lymph sacs. The specimens were fixed in Serra's solution (Serra, 1946), embedded in paraffin, sectioned serially and studied with the QH1 and QCPN antibodies as described below.

(B) In order to study the origin of the lymph hearts, somite 37 was grafted homotopically from stage 19 HH quail into chick embryos (9 experiments). After 6 days of reincubation, the specimens were fixed in Serra's solution, embedded in paraffin, sectioned serially and studied with the QH1 and QCPN antibodies as described below. Additionally, the morphology of normal lymph sacs was studied in hematoxylin and eosin (HE) stained paraffin sections and in semi-thin sections, according to standard techniques.

(C) The development of superficial, dermal lymphatics was studied by grafting of dermatomes of the thoracic region of stage 20 HH quail embryos into the wing bud region of day 3 chick embryos (12 experiments). After 5 days of re-incubation, the specimens were fixed in 4% PFA for 3 min, immersed in potassium phosphate buffer, 5% and 15% sucrose and embedded in tissue freeze medium. 20 μm sections were double stained with Prox1 and QH1 antibodies as described below.

In situ hybridization

Normal and experimental embryos were fixed overnight at 4°C in Serra's (1946) fixative. The samples were dehydrated, embedded in paraffin wax and 8 μm sections mounted onto silanized slides. The sections were deparaffinized, post-fixed in 4% paraformaldehyde solution (PFA) and, in older specimens, treated with proteinase K and refixed in 4% PFA. For the detection of cProx1 mRNA, a chick probe was used that has been described by Tomarev et al. (1996). The 1880-bp probe was cloned into pBluescript SK- and corresponded to positions 1442–3322 of the coding region of the *Prox1* gene. Linearization was performed with *Eco*RI (T3) and *Sac*I (T7) for the preparation of sense and anti-sense probes, respectively. For the detection of cTie2 mRNA, a chick probe was used that has been described (Jones et al., 1998). The probe was cloned into pBluescript SK. Linearization was performed with *Xho*I (T7) and *Not*I (T3) for the preparation of sense and anti-sense probes, respectively. Probe labeling was performed with digoxigenin RNA labeling kit as recommended (Roche, Mannheim, Germany). A hybridization mixture of 40% formamide, 25% 20× SSC, 1% Denhardt's solution, 1% tRNA, 1% herring sperm DNA, 2% labeled sense or anti-sense probe and 30% DEPC-treated water was prepared. Sixty microlitres 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 (Roche) in a blocking agent (1% bovine serum albumin in malate buffer) at 4°C overnight. The antibody was detected with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue-tetrazonium (NBT) (Roche) in alkaline phosphatase buffer, revealing a blue reaction product. The sections were counter-stained with nuclear fast red and the slides mounted. The sense controls did not reveal a specific signal.

For hybridization of whole embryos, specimens were fixed in 4% PFA, rinsed, dehydrated in 100% methanol and frozen. They were then rehydrated, treated with proteinase K, rinsed and post-fixed with 4% PFA. Hybridization was performed at 70°C overnight. Specimens were rinsed and incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody diluted 1:2000 in a blocking agent (Roche). The antibody was detected with BCIP/NBT in alkaline phosphatase buffer. The reaction was stopped with 1 mM EDTA in PBT, the specimens cleared in dimethyl formamide and stored in 4% PFA.

Immuno- and lectin-histochemistry

Endothelial cells of quail embryos or all quail cells were stained with the QH1 (Pardanaud et al., 1987) and QCPN antibodies, respectively

(Developmental Studies Hybridoma Bank, Iowa City, IA). Staining was performed according to the indirect peroxidase method as described previously (Wilting et al., 1995). The secondary antibody was peroxidase-conjugated goat-anti-mouse Ig (Sigma, Deisenhofen, Germany), diluted 1:300. Diaminobenci-dine (DAB) was used as a chromogen.

For staining of the media of vessels, an anti-smooth muscle α-actin (αSMA) antibody (Sigma) was used, diluted 1:5000. Secondary antibody and chromogen were the same as described above. Peroxidase-conjugated soya-bean agglutinin (SBA; Sigma) was dissolved in distilled water (50 μg/ml) and applied as a 1:4 dilution.

Immunofluorescence

For immunofluorescence studies specimens were embedded in tissue freeze medium (Leica). Cryosections of 20 μm thickness were prepared. Non-specific binding of antibodies was blocked by incubation with 1% bovine serum albumin (BSA) for 10 min. The QH1 antibody was diluted 1:100 and incubated with the sections for 1 h, as described previously (Papoutsi et al., 2000, 2001). After rinsing, the secondary Cy2- (green) or Cy3-conjugated (red) goat-anti-mouse antibody (Dianova, Hamburg, Germany) was applied at 1:100 for 1 h. Staining with polyclonal Prox1 antibody was performed as described previously (Wilting et al., 2002) at a dilution of 1:1000. The secondary antibody was Cy3-conjugated (red) goat-anti-rabbit antibody (Dianova, Hamburg, Germany). In the controls the primary antibodies were omitted. After rinsing, the sections were mounted under cover slips. They were then studied with an epifluorescence microscope (Leica, Bensheim, Germany).

Results

We have performed descriptive and experimental studies on the development of the lymphatic vascular system of chick and quail embryos. In histological sections, the most prominent anlage of the lymphatic vascular system is the paired jugular lymph sac (JLS), which can be easily observed in day 6.5 embryos. The JLS is located in the deep cervical region, dorso-laterally of the jugular vein (Fig. 1A). In quail embryos, the endothelial lining of the JLS is positive for the QH1 antibody (Fig. 1B). Smooth muscle cells, which in later stages can be found in lymphatic trunks, are not present in the JLS (Figs. 1C, D). In the quail, but not in the chick, the endothelial cells (ECs) of the JLS can be stained with soybean agglutinin. Veins are weakly positive, whereas arteries are negative (Figs. 1E, F). It has been shown previously, that the transcription factor Prox1 is constitutively expressed in lymphatic endothelial cells (LECs) but absent from blood vascular endothelial cells (BECs) of murine, avian and human tissues (Wigle and Oliver, 1999; Rodriguez-Niedenführ et al., 2001; Wilting et al., 2002). In day 6.5 chick and quail embryos, the JLS is Prox1-positive, and, like in the mouse (Wigle and Oliver, 1999), the adjacent segment of the jugular vein expresses Prox1 (Fig. 2A). In contrast, the endothelial angiopoietin receptor, Tie2, is expressed in the jugular vein but not in the JLS (Fig. 2B). By means of Prox1 in situ hybridization (ISH) it is possible to trace the anlagen of the lymphatics into earlier stages of development. In day 4 embryos, the jugular region is located around the level of somite 10 where the anterior and posterior cardinal veins fuse into the common cardinal vein. The vascular system of this region can be demonstrated with the endothelium-specific angiopoietin receptor Tie2 (Figs. 3A, B). Prox1 is not expressed in the blood vascular system, except for the jugular vessels (Figs. 3C–E). Prox1 positive cells can be seen in the lining of
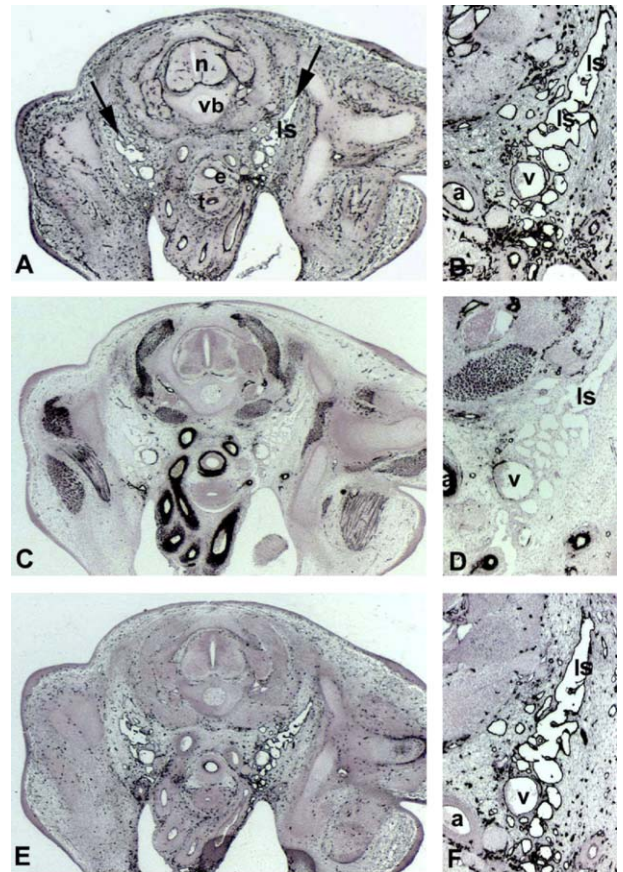

Fig. 1. Consecutive paraffin sections of a day 6.5 quail embryo demonstrating the position of the jugular lymph sacs (arrows). (A, B) QH1 antibody stains endothelial cells of all types of vessels like the aorta (a), jugular vein (v) and the jugular lymph sac (ls). (C, D) Staining with anti-α smooth muscle actin antibodies demonstrates the thick tunica media of the aortic arches (a) and other arteries, the thin tunica media of the jugular vein (v), and absence of smooth muscle cells from the jugular lymph sac (ls). (E, F) Staining with soybean agglutinin. The endothelium of the jugular lymph sac (ls) is positive, veins (v) are weakly positive and arteries (a) are almost negative. e, esophagus; n, neural tube; t, trachea; vb, vertebral body.

the anterior and posterior cardinal veins as demonstrated by ISH (Figs. 3C–E) and immunofluorescence (Figs. 4A–C). In quail embryos, this expression pattern is better visible than in chick embryos (compare Figs. 3D and E). In accordance with previous studies we have observed Prox1 expression in the neural tube, lens, retina and other neural placodes, as well as in the liver, heart, spinal, trigeminal and sympathetic ganglia. Furthermore, scattered Prox1-positive cells are found superficially in all somitic segments along the cranio-caudal axis (Fig. 3F). Immunofluorescence studies show that the cells are located in the dermatomes. A subpopulation of them is positive for the endothelial marker QH1 (Figs. 5A–D).

The origin of the JLS has not been studied in detail. Due to the expression pattern of Prox1, it has been suggested that in murine embryos the JLS develops by sprouting from the cardinal veins (Wigle and Oliver, 1999), but cell lineage studies have not been performed. Firstly, we have sought to determine the origin of the JLS from either the paraxial or the somatopleural mesoderm by grafting these mesodermal
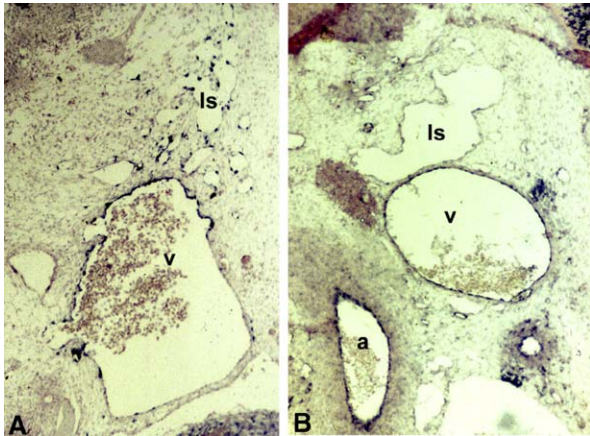
Fig. 2. (A) In situ hybridization (ISH) of a paraffin section of a day 6.5 chick embryo with Prox1 probe. The jugular lymph sac (ls) and a segment of the jugular vein (v) are positive. (B) Tie2 ISH of a nearby section. A signal can be observed in the aortic arch (a) and the jugular vein (v), but not in the jugular lymph sac (ls).

compartments homotopically from day 2 quail into chick embryos. Grafting of lateral plate shows that this tissue does not form any parts of the JLS (Figs. 6A, B). The somatopleura forms the scapula, body wall and limb structures, but not the endothelium of the JLS. Since we have shown previously that the lymphatics of the limbs are of somitic origin (Wilting et al., 2000), we determined if this also holds true for the JLS. Grafting of jugular somites shows integration of quail LECs into the superficial parts of the JLS, whereas the deep parts are formed by host (chick) LECs (Figs. 6C, D). In order to study the origin of the deep parts of the JLS we performed cell lineage studies with DiI-acLDL. Firstly we determined, if LECs possess functional LDL receptors by injecting DiI-acLDL directly into the lymphatics of the differentiated chorioallantoic membrane (CAM). The CAM possesses lymphatics, which accompany both arteries and veins. A few minutes after intra-lymphatic injection of DiI-acLDL a fine granular staining can be observed in the LECs, showing the existence of LDL receptors (data not shown). We then injected DiI-acLDL into a vitelline vein of day 4 quail embryos, before lymph sacs are present, and re-incubated the embryos until day 6.5. With the QH1 antibody the region of interest, i.e., aortic arch, jugular vein and JLS, can be identified (Figs. 7A, B). In the same section, the fine granular staining with DiI-acLDL can be seen in a number of LECs of the JLS (Figs. 7C, D). The data suggest a contribution of early venous ECs to the JLS.

We have observed Prox1/QH1 double positive cells in the dermatomes, which may represent lymphangioblasts. We tested their lymphangiogenic potential by grafting dermatomes of inter-limb-levels from day 4 (stage 20HH) quail embryos into the wing bud region of chick embryos. After 5 days of re-incubation, the lymphatics in the wing of the host embryos can be identified with the Prox1 antibody (Fig. 8A). The lymphatic vessels are located superficially in the dermis and are connected to the axillary region. Prox1 and QH1 double staining clearly shows integration of graft-derived quail LECs into the

lymphatics of the chick host (Figs. 8B–D), demonstrating lymphangiogenic potential of the dermatomes.

A highly specialized part of the superficial lymphatics of birds are the lymph hearts (Budras et al., 1987). They are located dorsal to the transverse processes of the first free caudal vertebra, which can be demonstrated in chick and quail embryos by injecting Mekox-blue into the umbilical lymphatics (Fig. 9A). The embryonic lymph hearts are partially covered by the *M. coccygeus dorsalis* (Fig. 9B). They are made up of an endocardium and a myocardium (Figs. 9C, D). The origin of the cells of the lymph hearts has not been studied yet. In day 5.5 chick and quail embryos, the anlagen of the lymph hearts can be detected with Prox1 ISH (Fig. 10A). A dense plexus of lymphatic capillaries is visible in the dermatomes of somites
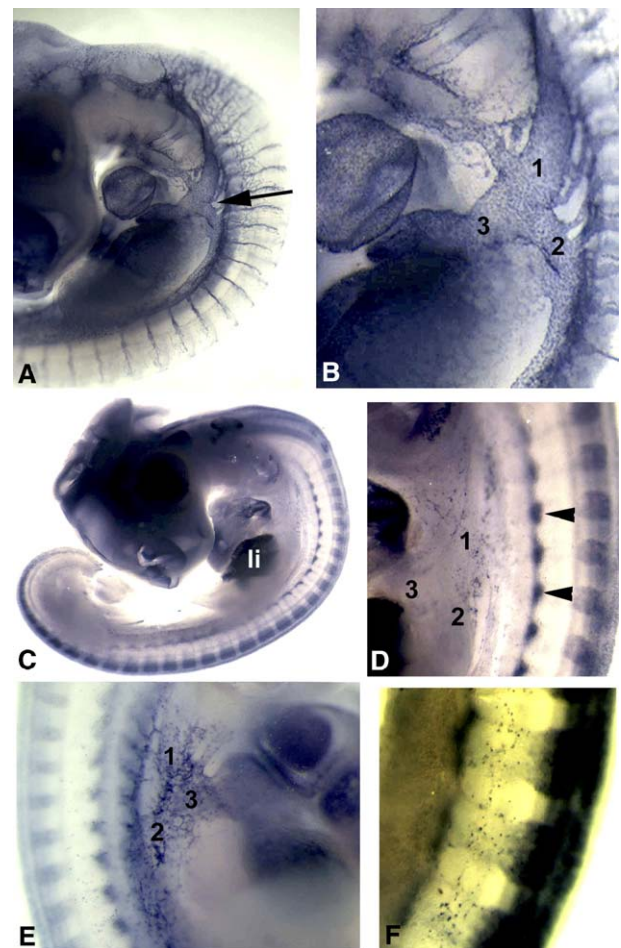

Fig. 3. Whole mount in situ hybridization of day 4 (stages 20–21HH) chick and quail embryos. (A) Chick embryo stained for Tie2. The jugular region (arrow) is characterized by the confluence of the anterior and posterior cardinal veins. (B) Higher magnification of (A) showing the confluence of the anterior (1) and posterior (2) cardinal veins into the common (3) cardinal vein. (C) Chick embryo stained for Prox1. Various organs are positive such as the lens, retina, ectodermal placodes, liver (li), heart, spinal, trigeminal and sympathetic ganglia. (D) Higher magnification of (C) showing the jugular region. Prox1 is expressed in cells, which seem to be located in the cardinal veins. Sympathetic ganglia (arrowheads). (E) Quail embryo stained for Prox1. The jugular section of the venous system is Prox1-positive. Anterior (1), posterior (2) and common (3) cardinal veins. (F) Higher magnification of the chick embryo shown in panel C. Scattered Prox1-positive cells are found in the dermatomes along the cranio-caudal axis.
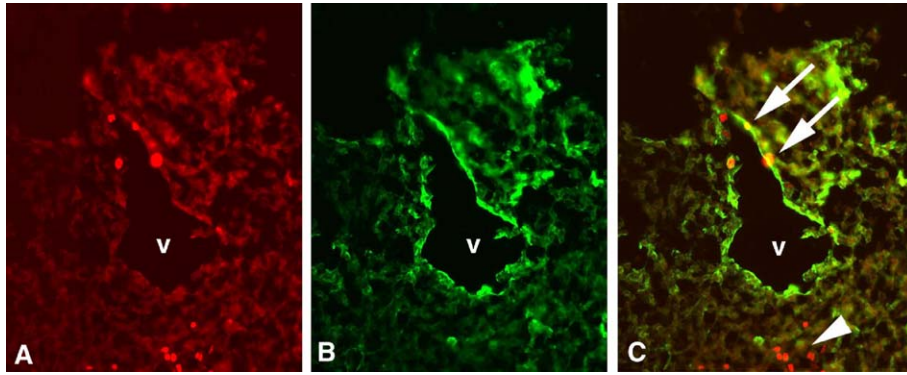
Fig. 4. Immunofluorescence double staining of a day 4 quail embryo (stage 20 HH) with Prox1 (A) and QH1 (B) antibodies. (C) Merged picture. Note the presence of Prox1-positive cells (arrows) in the jugular section of the cardinal veins (v). Other cells are Prox1-positive and QH1-negative (arrowhead).

35–40. Grafting of somite 37, which is in the centre of the prospective lymph heart, homotopically from quail into chick embryos, shows that both LECs and muscle cells of the lymph heart are of somitic origin (Figs. 10B, C). This could be demonstrated by staining of quail cells with the QCPN antibody. Staining with the QH1 antibody provided further evidence for the quail origin of LECs in the chimeras (Fig.
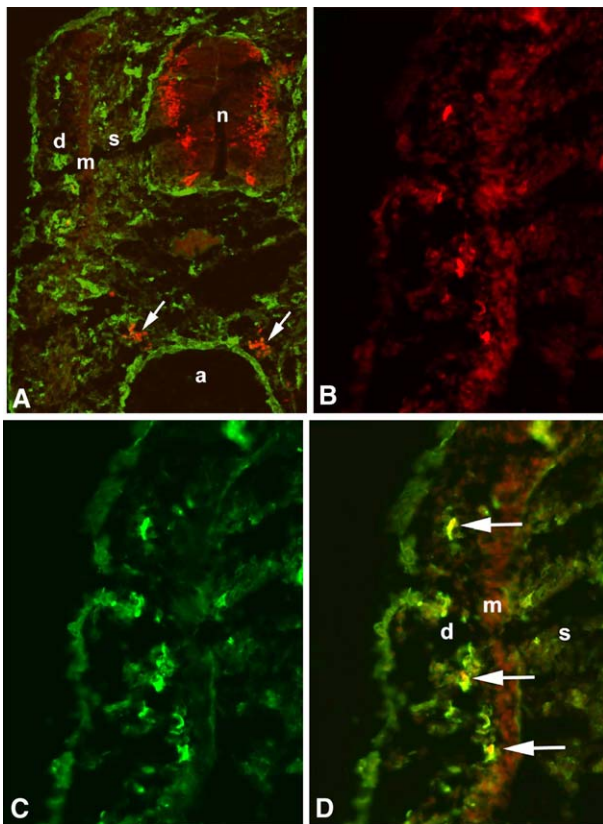

Fig. 5. Immunofluorescence double staining of day 4 quail embryos (stage 20 HH) with Prox1 and QH1 antibodies. Transverse sections of the thoracic region. (A) Overview showing Prox1 expression in the neural tube (n) and in the sympathetic ganglia (arrows) located close to the aorta (a). Dermatome (d), myotome (m) and sclerotome (s) are illustrated. (B) Prox1, (C) QH1 and (D) merged picture showing dermatome (d), myotome (m) and sclerotome (s) at higher magnification. Note that a subpopulation of cells in the dermatome is double positive (arrows).

10D). We counted the number of quail cells in the QCPN stained sections and found that approximately 18–20% of the cells were of quail origin, suggesting that five somites contribute to the lymph hearts.

Discussion

Development of lymphatics has been studied by injection methods, serial sections (Sabin, 1909; Clark, 1912a; van der Putte, 1975) and in living animals (Clark, 1912b; Clark and Clark, 1914). However, due to the lack of specific markers at that time, the origin of lymphatic endothelial cells had remained unknown. Are they derived from lymphangioblasts of the early mesenchyme (Huntington, 1908; Kampmeier, 1912), from veins by sprouting (Ranvier, 1895; Sabin, 1909), or by both mechanisms (van der Jagt, 1932)? Recently, highly specific markers have been found, which allow for discrimination between BECs and LECs. Among these markers is the homeobox transcription factor Prox1 (Wigle and Oliver, 1999). Prox1 is a stable and specific marker of lymphatics in normal and diseased human tissues (Wilting et al., 2002), and its essential role for the developing lymphatic system has been demonstrated in Prox1-deficient mice (Wigle and Oliver, 1999). Heterozygous Prox1 null-mice mice show accumulation of chyle in the intestine. Homozygous null-mice have almost no signs of lymphatic vessel development, whereas the blood vessels appear to be completely normal. As determined by Prox1 expression, development of the lymphatics starts around embryonic day (ED) 9.5 in the jugular region of wild-type mice (Wigle et al., 2002). Prox1 positive endothelial cells are seen in the jugular section of the anterior cardinal vein, and in ED 10.5 embryos it appears that Prox1-positive cells are sprouting into the mesenchyme. However, movement of cells has not been studied and cell lineage studies have not been performed in mice. In their studies on Prox1 expression in the lymphatic system, Wigle and Oliver (1999) describe their observation that during development "scattered (Prox1-positive cells) along the embryonic axis increased. It could be argued that this increase is a consequence of an independent induction of Prox1-positive cells in the adjacent mesenchymal cell. However, our results favor the idea that this increase reflected an augmentation in the number of cells budding from the cardinal vein." As a
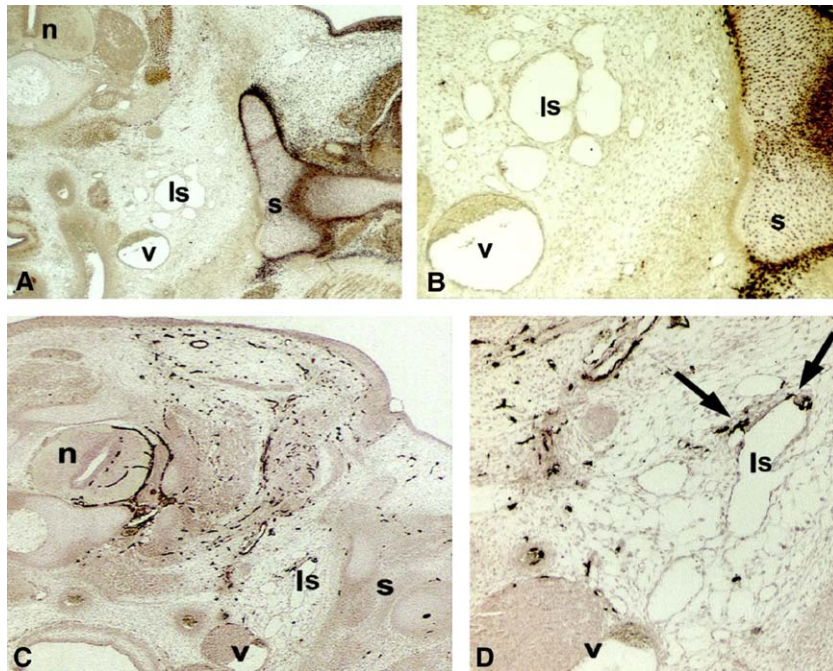
Fig. 6. Homotopical grafting performed on day 2 chick and quail embryos and re-incubation of the chick host until day 6.5. (A, B) Grafting of somatopleura and staining of all quail cells with the QCPN antibody. Nuclear QCPN staining shows that graft-derived quail cells form the scapula (s) and limb structures. The jugular vein (v) and lymph sac (ls) are negative. (C, D) Grafting of somite 10 and staining of quail endothelial cells with the QH1 antibody. The vessels of the body wall originate from the graft. There is integration of quail LECs into superficial parts (arrows in panel D) of the jugular lymph sac (ls). n, neural tube; s, scapula; v, jugular vein.
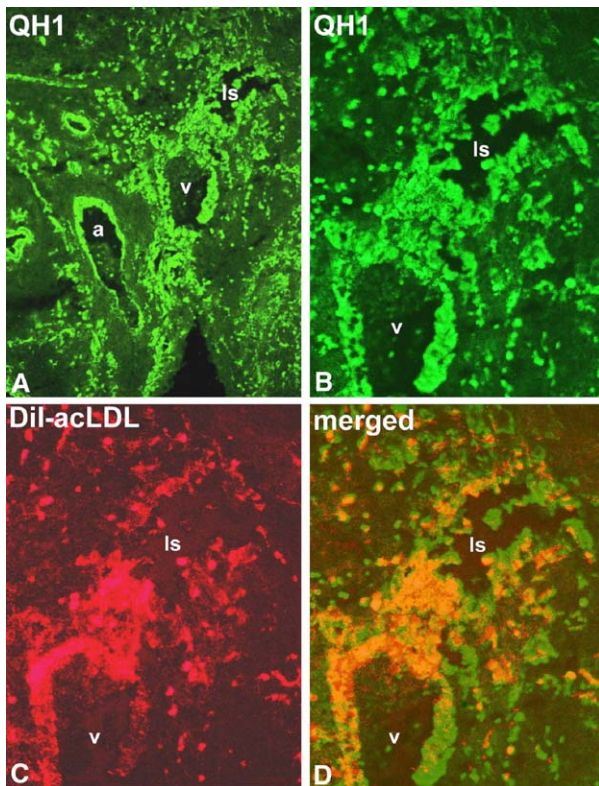

Fig. 7. Injection of DiI-acLDL into the vitelline vein of day 4 quail embryos. Re-incubation until day 6.5 and staining with the QH1 antibody (green). (A) Overview of the region of interest showing the aortic arch (a), jugular vein (v) and jugular lymph sac (ls). (B–D) Higher magnification of panel A. Note DiI-acLDL staining (red) in LECs of the JLS (yellow signal in the merged picture).

consequence, many original papers and reviews published in the recent years have concentrated on the idea that lymphatics develop from the venous system in a process of transdifferentiation of venous ECs into LECs (Wigle et al., 2002; Hong et al., 2004; Karkkainen et al., 2004).

Our grafting experiments and cell lineage studies performed on avian embryos show that the lymphatics have a dual origin. The Prox1 expression pattern in chick and quail embryos is the same as in the mouse. Besides expression in a number of non-endothelial cell types, Prox1 is a highly specific marker of LECs; the only exception being the concave side of the semilunar valves (Rodriguez-Niedenführ et al., 2001). Prox1 also is a marker of prospective LECs. It is expressed in the jugular section of the cranial and caudal cardinal veins (the region around somite 10 in day 4 avian embryos). These veins are Prox1 and Tie2 double positive whereas later the lymph sacs and intestinal lymphatics (data not shown) are Tie2-negative. Like in the mouse, the jugular veins seem to be the anlagen of the JLS. Our cell lineage studies performed with DiI-acLDL on day 4 quail embryos show that labeled venous ECs end up in the JLS. However, only the deep parts of the JLS seem to be derived from the veins whereas the superficial parts develop by integration of lymphangioblasts from the local somites. This is supported by a previous observation. When quail paraxial mesoderm is inserted superficially into the jugular region of chick embryos, integration of LECs into the superficial parts of the JLS can be observed. When grafted more deeply into the host, both the jugular vein and the lymph sac are made up of grafted cells (Wilting et al., 2001). Our data suggest that the superficial lymphatics are derived from local lymphangioblasts,
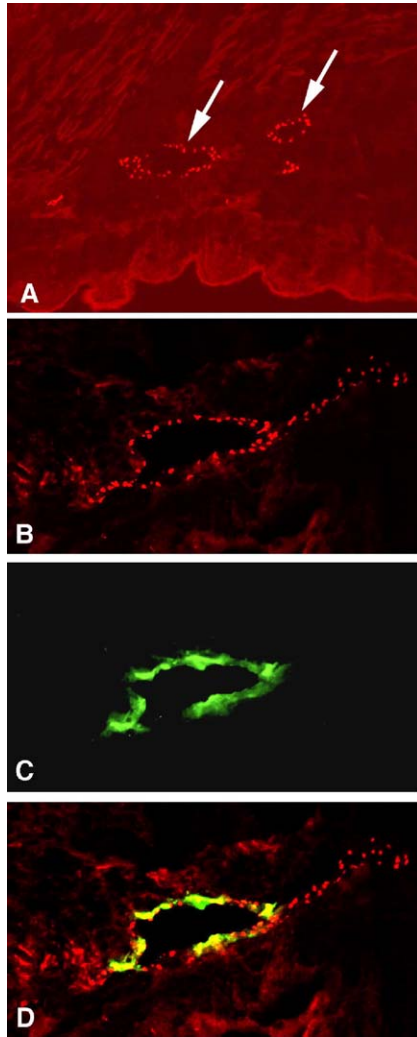
Fig. 8. Grafting of a single isolated dermatome of a stage 20 HH quail embryo into a day 3 chick embryo. Re-incubation period 5 days. (A) Overview showing Prox1-positive lymphatic vessels (arrows) in the dermis of the wing of the host embryo. Double staining with Prox1 (B) and QH1 (C). (D) Merged picture. Note integration of QH1-positive quail LECs into the Prox1-positive and QH1-negative lymphatic vessels of the chick host.

whereas the deep parts of the lymph sacs are formed by angioblasts of the paraxial mesoderm, which are incorporated into the jugular section of the cardinal vein, express Prox1 and sprout into the surrounding mesenchyme. This raises the question about the origin of the jugular veins. They are obviously not derived from angioblasts of the local somites, although it has clearly been shown that somites possess angiogenic potential (Wilting et al., 1995). However, extensive migration of angioblasts and endothelial cells occurs during the formation of trunk vessels (Wilting et al., 1995; Ambler et al., 2001), and it is likely that the jugular veins are initially formed at a different level in the early embryo, and are shifted by allometric growth toward the jugular region. Most likely the jugular veins are formed at a more cranial level since the heart undergoes a marked descent during development.

Our studies show that the superficial/dermal lymphatics are derived from local lymphangioblasts. We have observed scattered Prox1-positive cells in the dermatomes of day 4 avian embryos. These cells represent a heterogeneous population. Some of them may represent sympathetic neurons since the sympathetic ganglia are also Prox1-positive, others are Prox1/QH1 double positive. QH1 is a marker of BECs, LECs and angioblasts. Our grafting experiments show development of LECs from dermatomal precursor cells, most likely from the Prox1/QH1 double positive cells. The dermatomes are derived from the early epithelial somites. These are Prox1-negative (Tomarev et al., 1996), showing that there is a Prox1-negative phase of lymphangioblast development. In accordance, our previous studies have demonstrated that lymphatics of extra-embryonic sites develop independently from the lymph sacs. Grafting of the allantoic mesoderm of day 3 quail embryos into corresponding chick embryos has shown that the lymphatics of the chorioallantoic membrane (CAM) develop from precursors within this mesoderm (Papoutsi et al., 2001). By embryonic day 4, but not day 3, the precursors can be identified by their Prox1 expression, which again shows that there is a Prox1-negative phase of LEC development. In fact, the very early stages of lymphatic development can also be observed in Prox1 null-mice. Lymphatic development in these mice is arrested around ED 11.5 and the vessels appear to have a blood vascular phenotype (Wigle et al., 2002).

Very recent data suggest that lymphangioblasts cannot only be observed in avian embryos, but also in Xenopus laevis tadpoles and probably also in mice. Ny et al. (2005) report on clusters and cords of Prox1-positive cells in Xenopus tadpoles, which form the lumen of lymphatic vessels in later stages. During pathological, inflammation-induced lymphangiogenesis in the cornea of mice, CD11b-positive macrophages might transform into lymphangioblasts. In vitro, such cells are capable of expressing lymphatic endothelial markers and form tube-like structures (Maruyama et al., 2005).

The mechanisms of the formation of lymphatic vessels have not been studied in detail. One of these mechanisms seems to be sprouting (Karkkainen et al., 2004). In fact, the term sprouting has been introduced into lymphangiogenesis research some time ago by Ranvier (1895). In addition, formation of tubes by lymphangioblasts has been described very recently (Ny et al., 2005). We have shown that integration of lymphangioblasts into the lining of a lymphatic vessel is an additional mechanism, which is analogous to the growth of the blood vascular system. Another mechanism seems to be the fusion of lymphatic capillaries, which may take place during development of the lymph hearts. Lymph hearts are found in all avian embryos and are needed for the propulsion of lymph into the coccygeal veins. After hatching lymph hearts degenerate in some species, e.g. chicks, but remain active in birds that possess erectile copulatory organs, e.g., ducks (Budras et al., 1987). The lymph hearts are located superficially in the sacro-coccygeal region. In early embryos, a plexus of lymphatic capillaries can be observed in this region. This plexus spans from somite 35–40. The capillaries seem to fuse into a heart that possesses a single chamber, occasionally traversed by a trabeculum. After grafting of somite 37, only a small number (18–20%) of the lymph heart cells are of donor origin, suggesting that the other
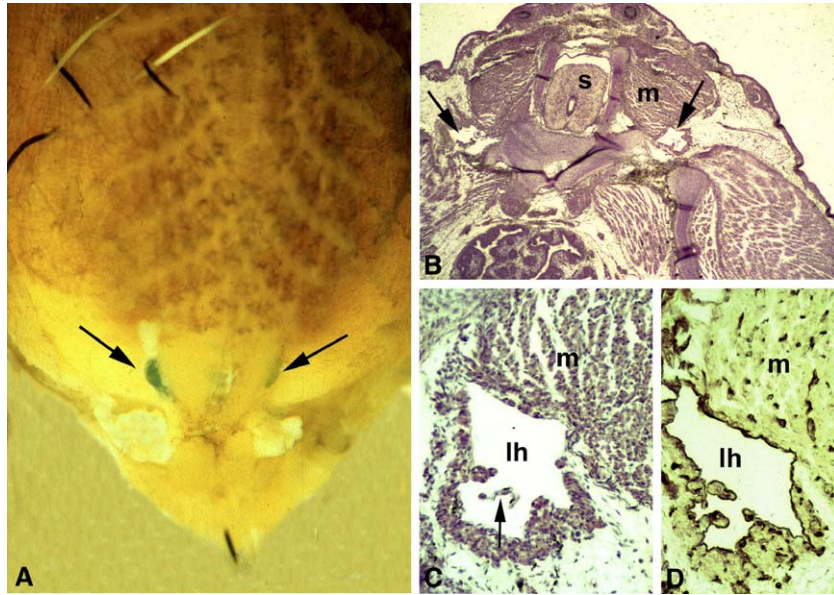
Fig. 9. (A) Merkox-blue injection into umbilical lymphatics of a day 14 quail embryo. After removal of the epidermis, the lymph hearts (arrows) can be seen in the sacro-coccygeal transitional zone. (B) Paraffin section of a day 12 quail embryo stained with hematoxylin and eosin. The lymph hearts (arrows) are located dorsal to the transverse processes. m, *M. coccygeus dorsalis*; s, spinal cord. (C) Higher magnification of B showing the muscular wall and a trabeculum (arrow) of the lymph heart (lh). (D) QH1 staining demonstrates the endocardium of the lymph heart.

parts are derived from neighboring segments. Both endocardium and myocardium of the lymph heart are of somitic origin. The myocardium is made up of striated muscle, which, however, is different from both cardiac and skeletal muscle (Budras et al., 1987). Nevertheless, the data show that all striated muscles, except for the blood heart, are derived from the paraxial mesoderm (Christ et al., 1998).

In sum, our studies show that the lymphatic vascular system of birds develops from two sources. The deep parts of the jugular lymph sacs are derived from the jugular segments of the cardinal veins. The superficial, dermal lymphatics are derived from local lymphangioblasts. The two compartments fuse to form the patent lymphatic system. Malformation of either the superficial or the deep compartment, or disturbances of the fusion of the two parts may result in lymphedema, which can be observed in the nuchal region of numerous genetically abnormal human fetuses.
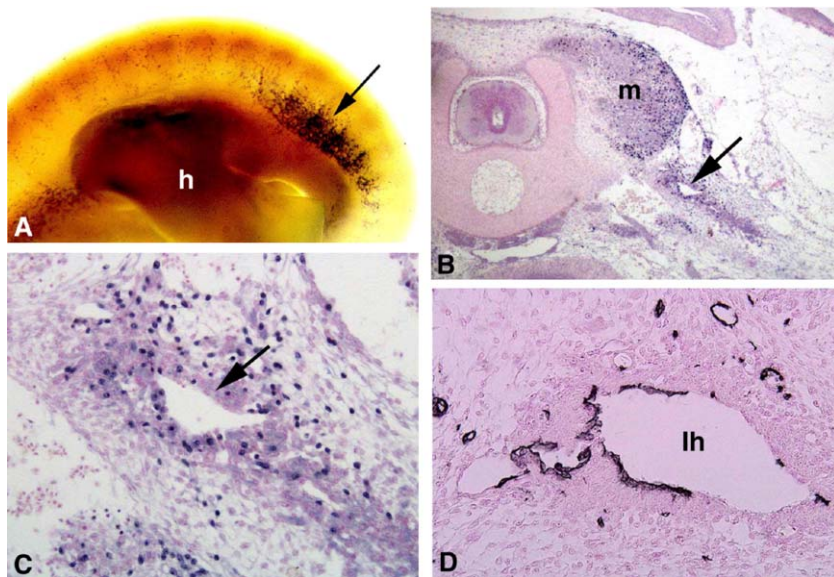

Fig. 10. Studies on the development of the avian lymph hearts. (A) Prox1 ISH of a day 5.5 chick embryo shows a plexus of lymphatic capillaries (arrow) in the sacro-coccygeal region. h, hind limb. (B–D) Homotopical grafting of somite 37 from a day 3 quail into a chick embryo. Re-incubation 8 days and staining of quail cell nuclei (black) with the QCPN antibody (B, C) and the QH1 antibody (D). Quail cells form both the endocardium and the myocardium of the lymph heart (arrow, lh). m, *M. coccygeus dorsalis*.

Acknowledgments

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