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THE LYMPHATIC VASCULAR SYSTEM: SECONDARY OR PRIMARY?

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ABSTRACT

It has generally been accepted that the blood vascular system is primary and the lymphatic vascular system secondary. Diseases of the blood vascular system are the leading cause for mortality and morbidity in developed nations. In contrast, lymphedema is seldom life-threatening and can generally be well-managed by combined physiotherapy. During ontogeny, the blood vessels and the heart develop much earlier than the lymphatic vessels. However, there is growing evidence that the first vascular system occurring during ontogeny and phylogeny has lymphatic functions. Defense mechanisms are crucial for all organisms irrespective of their size. Macrophages precede the emergence of erythrocytes during ontogeny, and their circulation in the hemolymphatic (more accurately, lymphohematic) system of insects, which do not possess erythrocytes, shows that the lymphatic function is primary whereas the nutritive function is secondary, needed only in larger organisms. In molluscs and arthropods, which have an open vascular system, hemocyanin has both oxygen transporting and defense functions. In vertebrates, the early blood vessels have structural characteristics of lymphatics and express the lymphendothelial receptor flt-4 (Vascular Endothelial Growth Factor Receptor-3). Later, flt-4 becomes restricted to the definitive lymphatics, which are either formed from the primary vessels or from mesodermal lymphangioblasts. The primary lymphatic function has become overruled by the nutritive function in blood vessels of larger animals. The circular movement of cells is driven by a blood heart, which, however, is not an unique organ. Lymph hearts are present in lower vertebrates, still develop transiently in birds, and are vestigial in the contractile lymphangion which "circulates" immune cells. We conclude that the definitive lymphatics are perhaps secondary in mammals, but the blood vascular system seems to develop on the basis of an ancestral lymphatic system with lymph hearts.

DEVELOPMENT OF THE VASCULAR SYSTEM: TRADITIONAL VIEW

The blood vascular system and circulation has been in the focus of scientific research ever since its discovery by William Harvey (1). On the other hand, the lymphatic system has mostly been neglected although the lymphatic vessels were discovered by Gasparo Aselli (2) at almost the same time. The predominant interest in the blood circulation compared to the lymph circulation is obviously due to its anatomic prominence and accessibility, critical nature of its functions, and the dramatic manifestations of its disorders as the leading cause for death in industrialized nations. Diseases of the lymphatic vessels are disfiguring; produce swelling, scarring and immune dysregulation, but are rarely lethal. The dense vascularization of tumors has been recognized even by Rudolf Virchow (3), and the intense tumor angiogenesis research of recent years is

mainly based on studies by Judah Folkman (4). Accordingly, the terms vasculogenesis and angiogenesis have almost without exception been used in the sense of hemangiogenesis (and blood vessel development and growth). Tumor-induced lymphangiogenesis has been neglected, even denied, but clear experimental evidence for this phenomenon has been published recently (5,6). The proper identification of lymphatic endothelial cells (LECs) in histological sections has been problematic, and only in the last few years have molecules been identified, which allow for more specific immunohistochemical staining of LECs (review: 7). There is not only a predominance of hemangiogenesis research over lymphangiogenesis research, there also is the generally accepted view that blood vessels are primary and lymphatic vessels secondary or an offshoot of blood vessels.

According to the biogenetic law of Ernst Haeckel, ontogeny is a recapitulation of phylogeny. During ontogeny, the circulatory system develops much earlier than the lymphatic vascular system. In the chick, the first blood vessels can be seen after one day of incubation (8), whereas morphological evidence for LECs is present around day 5 (9). In the mouse, blood vessel development starts at day 7.5 (10); the anlagen of the lymphatics can be seen in the jugular region at day 10 (11). In the human, jugular lymph sacs have been found in 6- to 7-week-old embryos of 10-14 mm total length (12). This is 3 to 4 weeks after the development of the first blood vessels. In sum, these findings seem to show that the blood vascular system is primary. Lymphatic vessels occur much later and are therefore secondary.

The nutritive function of the circulatory system is crucial for the development of vertebrates. Therefore, any mutation that affects the functionality of the cardiovascular system causes early embryonic death. Numerous genes with essential functions for the development of the circulatory system have been identified. These genes code for molecules that regulate cell proliferation as well as cell-cell and cell-matrix interactions. Among them are endothelial and endocardial growth factors and receptors such as Vascular Endothelial Growth Factors (VEGFs), their receptors (VEGFR-1,-2,-3; called flt-1, flk-1/KDR and flt-4, respectively) and co-receptors, the neuropilins (13, 14). The angiopoietins and their receptor, Tie2, as well as the orphan receptor Tie1 are of critical importance for embryonic hemangiogenesis (15), and, additionally, members of the ephrin ligand/Eph receptor family are needed for blood vessel formation (16). Mice with a deletion for vascular endothelial (VE)-cadherin die of vascular abnormalities (17), and the same is true for mice that lack extracellular matrix molecules that are critical for angiogenesis, such as fibronectin (review: 18). Abnormalities of the development of the lymphatic vessels are not embryonic lethal. Aplasia of lymphatic vessels is only found in severe genetic aberrations. Hypoplasia of lymphatics is found in children suffering from the Nonne-Milroy disease, and in some of the families, the inherited defect is a monoallelic mutation of the VEGFR-3 gene (review: 19). Mice lacking the transcription factor Prox1 have normal blood vessels but lack lymphatics (11). These mice die around embryonic day 14.5, but this seems to be due to the abnormal liver development. Therefore, there are many essential genes that control cardiovascular development, whereas development of lymphatics seems to be rather unproblematic and disturbed only in severe genetic aberrations.

Has nature created the highly problematic blood vascular system first, and only later developed a lymphatic system, which usually does not produce any serious developmental problems? Is it compatible with the genesis of higher organisms to produce a highly critical organ system out of zero, when almost every failure will be lethal? Wouldn't it be, from a teleological point of view, much more appropriate to make a critical developmental step on the basis of an already existing foundation?

NUTRITIVE OR IMMUNE FUNCTION: WHAT COMES FIRST?

It is generally assumed that the need for nutrition of all parts of the body has been the driving force for the evolution of the cardiovascular system. However, there is an excellent reason for its development even in small animals where nutrition is still by diffusion: this reason is immune defense. In fact, both phylogenetically and ontogenetically, defense mechanisms develop earlier than the nutritive elements of the blood. In *Drosophila*, supply with oxygen is accomplished by an elaborate system of fine channels, the tracheal system. Red blood cells are not present, and the open hemolymphatic system fulfils functions which are typical lymphatic functions. The heart is connected to open-ended vessels and pumps interstitial fluid and immune cells through the body. It is therefore more appropriate to call this system a lymphohematic or lymphatic system. Three types of immune cells are found in *Drosophila*: plasmatocytes, crystal cells and lamellocytes (20). Plasmatocytes, which are capable of phagocytosis, are comparable to the monocyte/macrophage lineage of the human. Crystal cells seem to have natural killer functions by producing free radicals via melanin synthesis. Lamellocytes differentiate in larvae, when these are attacked by parasites. They encapsulate the parasites and kill them by the activity of crystal cells. In adult flies, only plasmatocytes (phagocytes) are present. Like the other two cell types, they are produced during the larval period in so-called lymph glands, which are located pairwise along the dorsal vessel. Lymph glands originate from the lateral embryonic mesoderm. They differentiate in an anterior to posterior gradient and involute during aging. In older animals they cannot be found (21). This seems to be highly comparable to the involution of the lymphatic organs (bone marrow, thymus, lymph nodes) in aged humans. In *Drosophila*, plasmatocytes interact with the adipose body (liver-homologue) in the production of anti-microbial proteins. This shows that *Drosophila* has a well-elaborated innate immune system. The cardiovascular system of *Drosophila* ensures rapid circulation of immune cells through the body. By this means the cells are able to infiltrate a diseased body compartment much faster than would be the case by migration through the interstitium only. As in vertebrates, where lymph is composed of interstitial fluid and immune cells, the circulatory system of *Drosophila* and other insects has all the typical characteristics of a lymphatic system. In some invertebrates, this lymphatic system takes over an additional function, a nutritive one. Oxygen transporting molecules comparable to hemoglobin (arylphorin, hemocyanin) are present in various insects, arthropods and molluscs (22), but these molecules still have an additional defense function. In crustaceans, infection induces the cleavage of hemocyanin into antibiotic peptides (23). In cephalopods, hemocyanin binds to various particles and seems to act like an opsonizing agent, which increases the activity of phagocytes (24). This shows that the development of oxygen transporting molecules is closely linked to immunological mechanisms.

It is generally described that the first blood cells developing in vertebrates are of the erythropoietic lineage. In the chick, blood islands, which contain erythroblasts lined by an outer layer of endothelial cells, are seen in the yolk sac already after one day of incubation. The cells are derived from the posterior (extra-embryonic) mesoderm. This cell population is a transient one, and definitive hematopoiesis then takes place at intra-embryonic sites in the so-called AGM region (aorta, gonads, mesonephros) (review: 25). In mammalians including the human, the situation is a very similar one. Extra-embryonic blood islands can be seen in 13 to 15-day-old human embryos. The erythrocytes derived therefrom still contain a nucleus. The definitive hematopoietic cells develop from

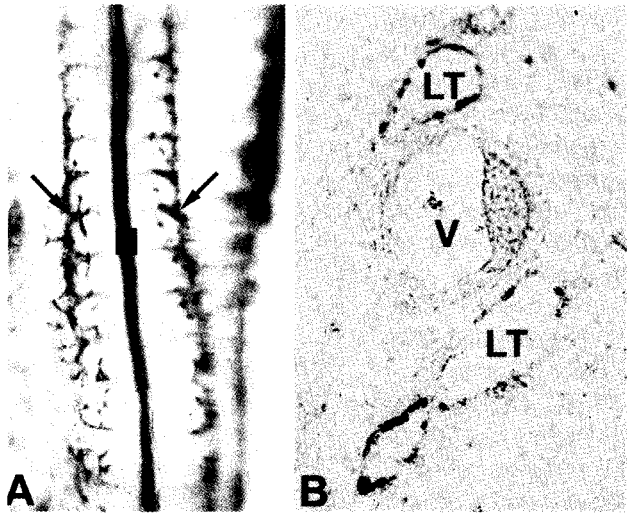

Fig. 1: A) In situ hybridization of a day 2 quail embryo with a VEGFR-3 anti-sense probe (kindly provided by Dr. Anne Eichmann, Paris). Ventral view. The developing aorta and intersomitic vessels (arrows) are positive. In the center, the notochord (N) also expresses VEGFR-3. B) In situ hybridization of a day 10 quail embryo with a VEGFR-3 anti-sense probe. The basilic vein (V) is negative, the accompanying lymphatic trunks (LT) are positive.

the splanchnic mesoderm. Mice that lack the transcription factor AML1 (Runx1) do not develop definitive blood cells (26). Successively, all blood cells are formed from pluripotent intra-embryonic stem cells. After the erythrocytes, the platelets can be found; then the neutrophilic granulocytes, thereafter the eosinophils followed by basophils and lymphocytes; and at last, monocytes are present in the peripheral blood (27). Therefore, it should be stressed that from these so-called blood cells only erythrocytes and platelets are by definition blood cells. The other cell types, (granulocytes, lymphocytes and monocytes/macrophages) are the free cells of the connective tissue, and only a very small minority of them is transiently found in the blood. Furthermore, it is important to recognize that the first "blood cells" in vertebrate embryos are not the erythrocytes. It is generally accepted that, in adults, monocytes are the precursors of macrophages, but in the embryo the situation is very different. Monocytes are the latest cells in the peripheral blood, but, in contrast, macrophages are the first immune cells found during development. They can be isolated from the epiblast before gastrulation, which is clearly before the development of blood islands (28). This situation is reminiscent of an innate immune function and seems to be fully comparable to the situation described for *Drosophila*. The fact that the transcription factor Runx1 is already active during the development of immune cells in *Drosophila* (20) suggests that the intra-embryonic hematopoiesis of vertebrates is based on ancestral mechanisms, whereas extra-embryonic hemangiogenesis is phylogenetically new. Therefore, as in insects, the first function of the intra-embryonic vascular system during ontogenesis of vertebrates may be defense rather than nutrition, the vessels being lymphatic, rather than blood vessels.

LYMPHATIC CHARACTER OF EARLY EMBRYONIC VESSELS

The suggestion that the first intra-embryonic vessels in vertebrate embryos are lymphatic vessels is also supported by their structure and by the expression of a typical

lymphendothelial receptor, VEGFR-3 (flt-4). Like lymphatic capillaries, embryonic vessels are not invested by a continuous basal lamina. They are extremely leaky and become tight at a time when definitive lymphatics develop. In chick embryos this occurs around day 5 (29). Uptake of interstitial fluid seems to be an important function of early embryonic vessels. Furthermore, the expression of VEGFR-3 (flt-4) in embryonic blood vessels is a strong indicator of their lymphendothelial function. In *Drosophila*, there is a single ancestor for the various VEGF and PDGF (Platelet-derived Growth Factor) receptors found in vertebrates. This receptor, called PVR, has three known ligands and directs embryonic immune cell migration (30-32). Probably only recently did these molecules assume their roles in vessel development, and it is conceivable that endothelial cells evolved from immune cells; again suggesting a primary defense function of the vascular system. In vertebrates, VEGFs and their receptors are essential regulators of blood and lymphatic vessel development. VEGF-A binds VEGFR-1 and -2, and is a key regulator of physiologic and pathologic blood vessel development (33). VEGF-C and -D bind VEGFR-2 and -3 and have been shown to induce lymphangiogenesis in various experimental settings (34). Therefore, VEGFR-3 is the receptor which is almost exclusively expressed on LECs, but not on BECs. However, this expression pattern holds true only for differentiated tissues. In mouse and chick embryos, VEGFR-3 is expressed in all endothelial cells during early development *(Fig. 1A, B)*, and becomes restricted to the lymphatics during the organogenic period (35, 36). VEGFR-3 knock out mice die from cardiovascular malformations before lymphatic vessels develop (37). This developmental regulation of VEGFR-3 expression has been interpreted to signify that lymphatics are derived from blood vessels. In contrast, we view this expression pattern as a reflection of the lymphatic functions of early embryonic vessels. These functions seem to become lost when the definitive lymphatics develop, and the cardiovascular system then becomes less permeable and focuses on its nutritive functions. Thus, the definitive lymphatics may be secondary but the early embryonic vessels have a primary lymphatic function.

The change from a primary lymphatic to a nutritive function of blood vessels may explain the difficulties in characterizing the different types of vessels in lower vertebrates, especially in fish, where many contradictory findings have been published. Mayer (38) tried to explain the different views about the piscine lymphatics by the existence of a secondary vascular system, a finding supported by Vogel and Claviez (39). The secondary vascular system constitutes a separate, parallel circulatory system and includes vessels earlier assumed to be lymphatics (40). It starts from the systemic arteries, forms its own capillary networks, which supply mainly the oral mucous membranes and the skin, and then returns to the systemic venous system. It is thought to function in skin respiration, osmotic regulation and immune defense. Based on its anatomical and functional characteristics, the hypothesis has been proposed that the secondary circulation might be an evolutionary predecessor of the lymphatic system (41). In contrast, one might suggest that this part of the vascular system has retained its original lymphatic character, and that the new, secondary lymphatics have not evolved in these fish. Ligands and receptors of the VEGF family that regulate blood vascular and lymphatic growth and development in higher vertebrates are also present in fish (42) but their relevance for the secondary vascular system has not been analyzed.

BLOOD HEART AND LYMPH HEART

One may argue that the driving force (*vis a tergo*) behind the perfusion of the vascular system is the heart, and that the heart is a typical feature of the blood vascular system. However, lymph hearts are

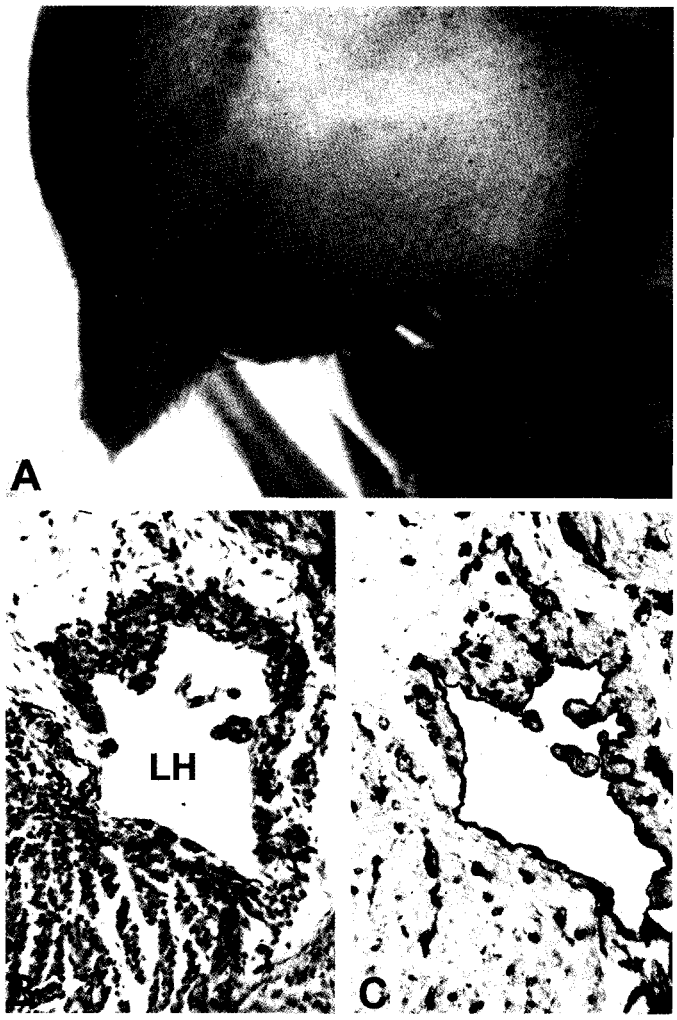

Fig. 2: A) Injection of Merkox-blue into an umbilical lymphatic trunk of a day 16 chick embryo. The lymph hearts (arrow) show up in blue. They are located superficially at each side of the vertebral column at the sacro-coccygeal transition. B) Hematoxylin and eosin staining of a lymph heart (LH) of a day 14 quail embryo. It consists of an inner endothelial lining and a layer of myocytes. A trabeculum transverses the lumen. C) Consecutive section of the lymph heart (LH) shown in B. The endothelial cells are stained with the QH1 antibody (8).

characteristic organs of lower vertebrates (43). Similarly to the blood heart, which is located at the interface between veins and arteries, the lymph hearts are situated at the transition from lymphatic vessels to the veins. Lymph hearts can generally be found in vertebrates in three different areas: jugular, lumbar and caudal. The amphibian lymphatic system mostly communicates with the venous system in all of these areas, and lymph hearts range in number from four or six in frogs to more than two hundred in caecilians. The important function of lymph hearts is to maintain the directed lymph flow and entry of lymph into the blood circulation. In reptiles, lympho-venous anastomoses exist in the caudal and the jugular regions but lymph hearts are found

only in the caudal region (40). Mammals and most birds do not possess lymph hearts but in birds these are still found as transient embryonic organs *(Fig. 2)*. The structure of the lymph hearts of birds is very similar to that of the blood heart. They have an inner endocardial lining and a contractile myocardium composed of striated muscle cells. Trabeculae can be found as in the blood heart as well as a valvular system (44). This finding shows that a heart is not restricted to the blood vascular system, and it cannot be excluded that the blood heart has evolved on the basis of a lymph heart. In fact, in *Drosophila*, the heart and the immune cells develop from a pool of mesodermal precursors, which are characterized by the expression of the gene *odd* (45). A subpopulation of cardiac cells seems to be closely related to the lymph glands, suggesting, again, a primary lymphatic function of the circulatory system. In the human, the spontaneous, peristaltic contractions in the tunica media of lymphatic trunks, generated by pacemaker cells, is highly reminiscent of the cardiac physiology. Usually, 8 to 10 contractions per minute can be observed (46) and respond to stretch and filling volume.

CONCLUSION

We conclude, that the separation of the human vascular system into a primary blood vascular system and a secondary lymphatic vascular system is not appropriate (based on the evidence). The situation is complicated by the fact that the blood vessels and the blood heart have developed on the basis of a primary lymphatic function of the system. The nutritive function seems to be secondary and has eclipsed the lymphatic function during phylogeny. Embryonic blood vessels still possess lymphatic functions, and only the definitive lymphatics may be secondary. They are produced by the activity of transcription factors such as Prox1, which is expressed in specific sites of veins and in mesodermal precursor cells (11,47). Häckel's view of the recapitulation of phylogeny during ontogeny may still be applicable to the vascular system, but the starting point for vessel development may be different from what has been previously suggested: residing in small animals with primary defense functions of the vascular system.

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