VEGFR-3 Ligands and Lymphangiogenesis

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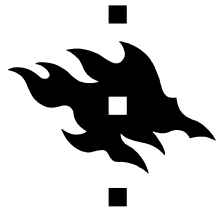

Academic dissertation

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Abbreviations

Akt	Oncogene from AKR thymoma mouse
Bcl-2	B-cell lymphomal leukemia oncoprotein
BR3P	Balbiani ring 3 protein
CAM	Chorioallantoic membrane
E	Embryonic day
EpoR	Erythropoietin receptor
FGF	Fibroblast growth factor
FOXC2	Winged helix/forkhead transcription factor subclass C member 2
Grb2	Growth factor receptor-bound protein 2
kDa	Kilodalton
MAPK	Mitogen-activated protein kinase
OMIM	Online Mendelian inheritance in men
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PI3-K	Phosphatidyl inositol-3-kinase
PKC	Protein kinase C
PLCγ	Phospholipase C-gamma
PlGF	Placenta growth factor
Ras	p21 oncoprotein from rat sarcoma
Shc	Src homology and collagen domain
STAT	Signal transducer and activators of transcription
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VHD	VEGF homology domain

List of Original Publications

This thesis is based on the following articles, which will be referred to by their Roman numerals.

I. Jeltsch, M.*, Kaipainen, A.*, Joukov, V., Meng, X., Lakso, M., Rauvala, H., Swartz, M., Fukumura, D., Jain, R. K. and Alitalo, K. (1997): **Hyperplasia of lymphatic vessels in VEGF-C transgenic mice** [published erratum appeared 1997 in Science 277, 463]. *Science* 276, 1423-1425.

II. Oh, S. J., Jeltsch, M. M., Birkenhager, R., McCarthy, J. E., Weich, H. A., Christ, B., Alitalo, K. and Wilting, J. (1997): **VEGF and VEGF-C: specific induction of angiogenesis and lymphangiogenesis in the differentiated avian chorioallantoic membrane**. *Developmental Biology* 188, 96-109.

III. Achen, M. G., Jeltsch, M., Kukk, E., Makinen, T., Vitali, A., Wilks, A. F., Alitalo, K. and Stacker, S. A. (1998): **Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4)**. *Proceedings of the National Academy of Science of the USA* 95, 548-553.

IV. Jeltsch, M., Karpanen, T. and Alitalo, K. (manuscript submitted, 2002): **Identification of VEGF receptor specificity determinants using VEGF - VEGF-C mosaic molecules**.

*These authors contributed equally to this work.

Abstract

Most of us have seen their own blood at one or the other time. The occasion might have been a small accident or in unfortunate cases a severe blood loss caused by a major injury. We also can feel our heart beating and the resulting pressure wave, the pulse. The existence of the cardiovascular system is obvious to us. Unlike the cardiovascular system, the lymphatic system has, until recently, escaped notable attention not only by the laymen, but also by the scientific community. It is unclear why the lymphatic system originally developed in higher vertebrates. Now, its main function seems to be to collect fluid that has leaked from the blood vessels and to return it into the cardiovascular system. Much of our knowledge about the development and structure of the lymphatic system is of considerable age, and it has been said that there has not been any progress in our understanding since the fine structure of the lymphatics was described with the introduction of the electron microscope.

Vascular endothelial growth factor (VEGF) is the principal direct inducer of blood vessel growth, but it does not promote the growth of lymphatic vessels. This study demonstrates for the first time specific lymphangiogenesis as a response to the VEGF homologue VEGF-C. Overexpression of full length VEGF-C under the keratin-14 promoter in the skin of transgenic mice caused a proliferation of the lymphatic endothelium and lymphatic vessel enlargement. In the chorioallantoic membrane assay, the mature form of VEGF-C was also largely specific for lymphatic endothelial cells. A newly discovered close homologue of VEGF-C, VEGF-D was then shown to have the same receptor-binding pattern as VEGF-C.

Contrary to the interaction of VEGF with its receptors, VEGF-C interaction with VEGFR-3 has not been analyzed at the molecular level. The structural determinants of VEGFR-3 binding were characterized in relation to VEGF using a non-random family shuffling approach with VEGF and VEGF-C as parent molecules. This approach led to the identification of VEGF/VEGF-C mosaic molecules that showed novel receptor binding profiles and a panel of these molecules was used to delineate the requirements of specific receptors in the induction of angiogenesis versus lymphangiogenesis.

Review of the Literature

1 The Two Vascular Systems

Large multicellular organisms with a high metabolic demand use carrier molecules to distribute oxygen within their bodies. In vertebrates a closed vascular system guides the transport of these carrier molecules. Therefore the vascular system has to be functional early in development. When the human embryo reaches the size of approximately 3 mm at embryonic day 22, its heart starts beating. Later, when the cardiovascular system is already functioning, a second vascular system develops: the lymphatic system. Unlike the cardiovascular system it is not a circulatory system. Lymphatic flow starts in blind-ended capillary networks that penetrate most of the body tissues. Collecting lymphatics drain the capillary networks and after collecting fluid from many tributaries the largest collecting lymphatic vessel (the thoracic duct) ultimately reaches the veins. A schematic view of the general setup of the lymphatic system is given in Figure 1.

Compared to our tremendous knowledge about the cardiovascular system the understanding of the lymphatic system is still very rudimentary. The lymphatic system has lagged behind the cardiovascular system both in its discovery and exploration, probably due to the cardiovascular system's visual prevalence and importance.

Retrospectively, three heydays have shaped our understanding of the lymphatic system. The first took place during the first years of the 20th century when researchers like Sabin, Kampmeier, Huntington and McClure were studying the ontogeny of the lymphatic system (reviewed by Wilting et al. 1999). The second boost of knowledge resulted from the use of the electron microscope during the 1960s to solve questions about the fine structure of the initial lymphatics and their functioning (reviewed by Leak 1970). At the moment, lymphatic research is experiencing an impressive comeback thanks to the arrival of molecular biology, genetics and, last but not least, by the discovery of markers specific for lymphatic endothelium (reviewed by Oliver and Detmar 2002).

2 The Cardiovascular System

During early embryogenesis, most of the blood vessels form by *vasculogenesis*, the in-situ formation of an immature network of endothelial channels by the differentiation of precursor cells (*angioblasts*). Vasculogenesis starts in extra-embryonic tissues where putative mesodermal precursor cells (*hemangioblasts*) aggregate into blood islands. Cells in the center of a blood island develop into hematopoietic stem cells, and cells at the periphery differentiate into angioblasts. In the embryo proper, vasculogenesis gives rise to the heart endocardium, the paired dorsal aortas and the primitive vasculature of endodermally derived organs (e.g. lung, spleen and pancreas; reviewed by Wilting and Christ 1996).

The primitive vascular network grows and remodels into a functional, hierarchical structure containing large caliber vessels for low-resistance fast flow and small capillaries optimized for diffusion. Different cellular and molecular mechanisms (splitting, fusion, sprouting, and intercalation) participate in the remodeling and expansion and they are collectively referred to as *angiogenesis* (reviewed by Risau 1998). While most organs become vascularized by a combination of vasculogenesis and angiogenesis, initially avascular ectodermal tissues such as the brain, become vascularized exclusively by angiogenic mechanisms (Plate 1999).

The endothelial cell layer (*intima*) of vessels larger than capillaries becomes wrapped by additional sheets: a cover of muscular tissue

(*media*) and connective tissue (*adventitia*). Therefore, arteriogenesis requires the recruitment and organization of non-endothelial cells (reviewed by Carmeliet 2000a). Recent data shows that during embryonic development blood vessels not only fulfill transport functions but also play important roles in the induction and morphogenesis of organs (Lammert et al. 2001; Matsumoto et al. 2001). It has been thought that angiogenesis is the only way of neovascularization in adult organisms, but recently a population of progenitor cells able to differentiate into endothelial cells was isolated from circulating blood (Asahara et al. 1997) and identified as originating from bone marrow (Shi et al. 1998). Although the evidence for vasculogenesis from circulating endothelial precursors is convincing, the relative contribution of endothelial precursors to adult physiological or pathological angiogenesis is still unclear (Asahara et al. 1999; Crosby et al. 2000). In any case, there is reason to rigorously review these reports, since it has been shown that cell fusion events can produce experimental results that can be misinterpreted as differentiation events (Terada et al. 2002).

Quiescence is the default state of adult vasculature, and only few physiological processes in the adult involve endothelial cell proliferation, e.g. the female reproductive cycle (reviewed by Reynolds et al. 1992) or exercised-induced muscular hyperplasia (reviewed by Tomanek and Torry 1994). On the other hand, in disease angiogenesis frequently contributes to the pathological process (e.g. in tumorigenesis) and even may be the main pathological process itself (e.g. in wet age-related macular degeneration; reviewed by Carmeliet and Jain 2000).

Vascular endothelium is heterogeneous. Three distinct types of blood capillaries can be distinguished: continuous, fenestrated and discontinuous. Continuous endothelium has an uninterrupted basement membrane and most of the capillaries belong to this type. Fenestrated endothelium is characterized additionally by the presence of circular transcellular openings (*fenestrae*) with a diameter of 60 to 80 nm. Fenestrated endothelium is usually found in organs with high rates of fluid exchange (small intestine, kidney, salivary glands). Discontinuous endothelium has large intercellular gaps (up to 1 μm) and no basement membrane, and allows for almost unrestricted transport of molecules from interstitium to capillary lumen. Discontinuous endothelium is only found in specialized organs, e.g. in the liver, spleen and bone marrow (reviewed by Risau 1998).

3 Form and Function of the Lymphatic System

The lymphatic system consists of a network of thin-walled vessels that drain fluid and particular matter from the interstitial spaces. However, unlike blood vessels, the lymphatics do not form a circular system. The unidirectional lymph flow recovers fluid from the periphery and returns it to the cardiovascular system (see Figure 1).

Lymphatic flow begins in the capillary networks (*initial* or *terminal lymphatics*). Lymphatic capillaries consist of a single layer of non-fenestrated endothelial cells sitting on an incomplete basement membrane. Prenodal collecting vessels drain the capillary networks and transport lymph to the regional lymph nodes. Lymphatic capillaries are not invested with mural cells, but collecting vessels do posses a smooth muscle cell layer (Schmid-Schonbein 1990b). Postnodal collecting vessels carry lymph between successive sets of lymph nodes or to larger lymphatic collecting vessels. Eventually, the larger lymph-collecting vessels drain lymph from the final set of lymph nodes into the lymph ducts. Lymph from the intestinal, hepatic and lumbar region drains into the cisterna chyli. The cisterna chyli acts as a collecting reservoir at the posterior end of the thoracic duct. The thoracic

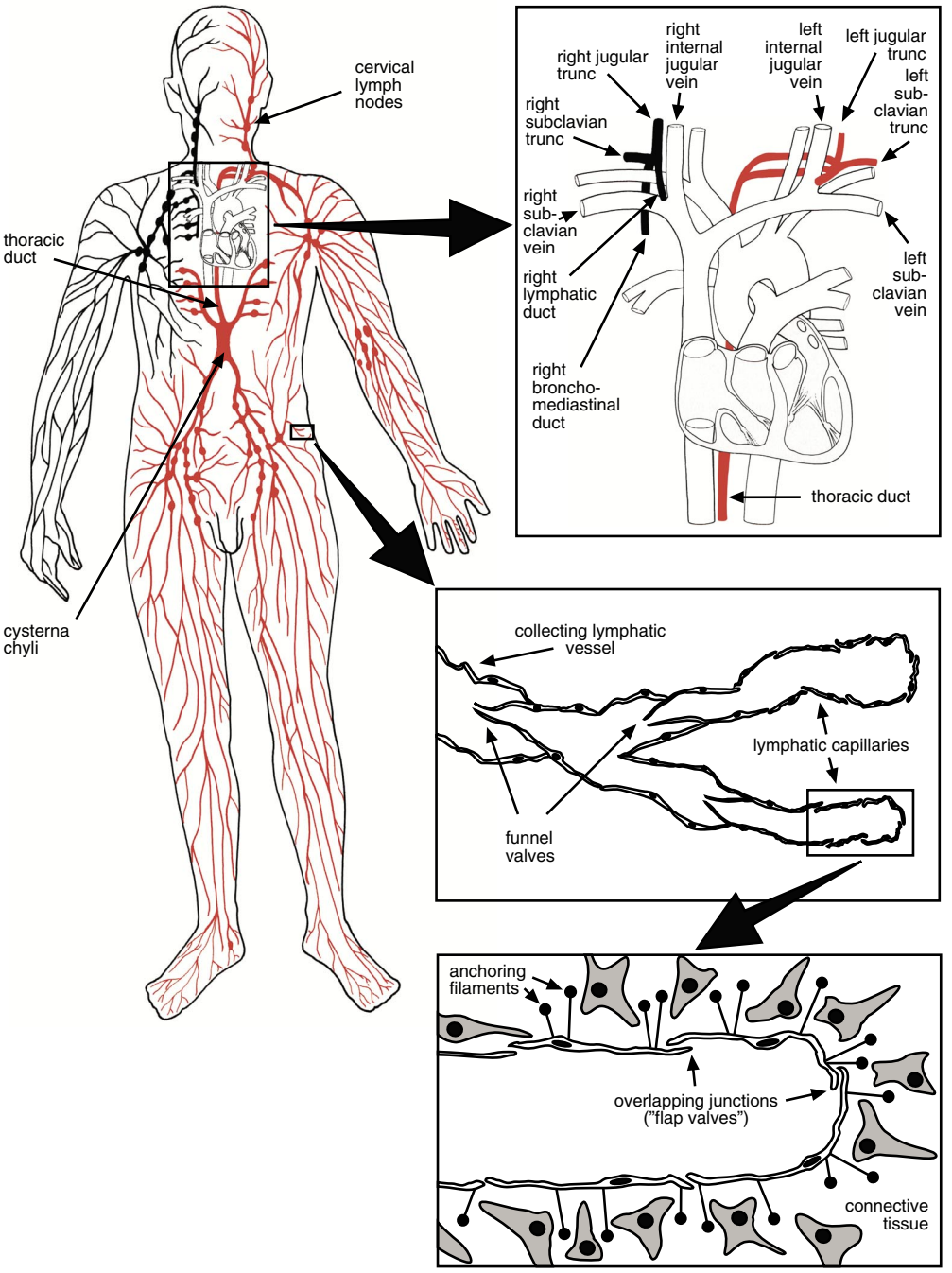

Figure 1. Schematic view of the lymphatic system

(adapted from Klein 1990)

duct ascends upwards from the cisterna chyli. On its way it receives lymph from the rest of the body except from the upper right quadrant. The thoracic duct empties directly into the venous blood at the junction of the left internal jugular vein and the left subclavian vein. The lymph of the upper right quadrant is collected into the right lymphatic duct and returned into the veins at the right jugulo-subclavian confluence (for a general review of the anatomy and the function of the lymphatic system see Foster 1996; Swartz 2001).

3.1 Drainage

The heart is a powerful pump, and when blood enters the capillary bed it is still under a pressure of about 4-5 kPa. Due to this pressure 20 to 30 liters of plasma leak each day from the capillaries and become interstitial fluid (Landis and Pappenheimer 1963). The orthodox view is that about 90% of this extravasated fluid is reabsorbed at the venous end of the capillaries and post-capillary venules, driven by osmotic forces (Starling 1895-96). The remaining 10% are drained by the lymphatic vessels and returned to the cardiovascular system. For this reason lymphatic capillary beds are found in most vascular tissues. It should be noted, however, that not all experimental data supports the reabsorption theory (Bates et al. 1994). Two to five liters of thoracic duct lymph are formed in humans each day (Bierman et al. 1953; Linder and Blomstrand 1958). Lymph contains most of the components of plasma, although the concentration of high molecular weight components is lower due to the capillary filtration effect. The total protein concentration is about two thirds of that of serum (Bergstrom and Werner 1966; Werner 1966).

3.2 Immune Defence

In addition to the drainage function, the lymphatics play multiple important roles in immune defence mechanisms. If body tissues are invaded by pathogens some are taken up together with the interstitial fluid by the lymphatics. The lymph passes through one or several lymph nodes before it enters the venous system. In collaboration with the antigen-presenting cells of the lymph nodes, T and B cells recognize non-self epitopes and mount an immune response. The activated immune cells proliferate in the lymph nodes and produce antibodies. Both cells and antibodies are delivered into the general circulation via the lymphatics. Not only the interstitial spaces but also the blood is screened via the lymph nodes, since about 50% of the plasma protein passes through the lymphatics each day (Klein 1990).

3.3 Intestinal Absorption

Another significant function of the lymphatics is the intestinal absorption and transport of long chain dietary triglycerides and lipophilic compounds such as fat-soluble vitamins or chlorinated organic compounds. After absorption from the gastrointestinal tract, blood and lymph capillaries compete for the transport of molecules to the systemic circulation. The majority of enterally administered compounds is absorbed into the portal blood since its throughput is about 500 times higher than that of the lymph. However, high molecular weight molecules and colloids are preferentially taken up by the lymphatics because of the highly permeable structure of the intestinal villous lymphatics (*chylous vessels* or *lacteals*). E.g. chylomicrons have a diameter of 200 to 800 nm and after being assembled and released by the enterocyte, gain access almost exclusively to chylous vessels. When pharmacological substances are absorbed by intestinal blood capillaries and transported via the portal blood to the liver, a large proportion can become inactivated (*first-pass metabolic effect*). Intestinal lymphatic absorption bypasses first-pass metabolic effects (Porter 1997).

3.4 Lymph Formation

The endothelial cells of the initial lymphatics lack tight junctions. Instead they are equipped with overlapping endothelial junctions, which function as mechanical flap valves. Filaments anchor the lymphatic endothelial cells into the surrounding connective tissue and are involved in the operation of the valves. Increased interstitial pressure forces these inter-endothelial valves to open and interstitial fluid and particulates gain access to the lymphatic lumen (Casley-Smith 1980).

In addition to fluid uptake via the valves, fluid transport by transcytosis and transendothelial channels plays a role (Leak 1971). Also both the hydrostatic and osmotic pressure gradient between lymphatic lumen and the interstitium have been suggested to contribute to lymph formation, although these forces cannot account for the removal of large proteins and particulate matter (Casley-Smith 1982a). The relative contributions of these mechanisms varies, and the mechanical valves seem to be especially important in situations of increased functional demand.

3.5 Lymph Propulsion

Lymphatic capillaries have no intrinsic contractility and depend entirely on extrinsic forces for lymph propulsion. The notable exception are the initial lymphatics in the bat wing, which do have their own contractile smooth muscle (Hogan and Unthank 1986). Alternating compression and dilation of the lymphatics by respiratory movement, contraction of skeletal and intestinal muscles, and the pressure pulse generated by adjacent arteries and arterioles propels lymph forward (Schmid-Schonbein 1990a). The directional flow of the lymph is maintained by funnel-shaped valves. The unit between two valves in the collecting lymphatics is termed lymphangion.

Contrary to the initial lymphatics, collecting lymphatics are contractile. Lymph flow from one lymphangion to the next is maintained against a pressure gradient and both extrinsic forces and contractions of the smooth muscle cell layer work together to pump the lymph against this gradient (Kinmonth and Taylor 1956; Olszewski and Engeset 1980). Median pressure in the initial lymphatics is close to atmospheric or interstitial values (Zweifach and Prather 1975). The pressure rises in the collecting lymphatics, and in the thoracic duct diastolic pressure ranges between –0.7 and 2.3 kPa and systolic pressure between 0.3 to 3 kPa (Kinnaert 1973).

3.6 Pre-lymphatic Channels

Interstitial transport happens both by diffusion and convection. Movement of large molecules and particles in the interstitium is not always uniform (Jain and Gerlowski 1986), suggesting the existence of preferred pathways ("pre-lymphatic tissue channels"; Casley-Smith 1980), but the significance of these channels and even their existence has been questioned (Casley-Smith 1982b). Drainage can be achieved via "pre-lymphatic channels" as seen in the central nervous system: there is little doubt about the quasi-lymphatic function of the "pre-lymphatic" perivascular spaces in the brain. These spaces (*Virchow-Robin spaces*) connect to the cervical lymph nodes (Casley-Smith et al. 1976), and this connection is important for both drainage and immune response to brain infections (reviewed by Esiri and Gay 1990; Weller et al. 1996). Based on these functional criteria these channels have been occasionally classified as lymphatic, although they are devoid of an endothelial lining. However, capillary filtration is greatly reduced in the brain due to the blood brain barrier, and the majority of drainage is accomplished via the cerebrospinal fluid by the arachnoid villi and the adjacent specialized venous sinuses of the dura (Weed 1922/1923). There are several other vascular

structures whose classification based on immunohistochemical and functional criteria remains inconclusive. These structures include the canal of Schlemm in the eye (Foets et al. 1992) and the perivascular spaces of the liver. It has been shown that genes specific for lymphatic endothelial cells, e.g. the receptor tyrosine kinase VEGFR-3, can be upregulated in non-lymphatic endothelial cells that fulfill a lymphatic function (Partanen et al. 2000). In the liver both blood vascular endothelial cells and hepatocytes line the perivascular spaces of the discontinuous liver capillary endothelium (*Spaces of Disse*). Despite little evidence they are assumed to fulfill a draining function, especially since the liver lobules themselves do not contain lymphatics (Niiro and O'Morchoe 1986; Trutmann and Sasse 1994). Also non-endothelial cells with an endothelial function have been shown to express VEGFR-3, e.g. the trophoblast cells of the placenta (Dunk and Ahmed 2001).

The matrix-lined channels seen in some melanomas are reminiscent of pre-lymphatic tissue channels. It was suggested that these channels participate in the tumor circulation, and such a behavior has been termed *vasculogenic mimicry* (Maniotis et al. 1999; Folberg et al. 2000). Tumor cells are known to contribute to the intima of tumor vessels, but the absence of endothelial cells in vasculogenic mimicry as described by Maniotis et al. remains controversial (McDonald et al. 2000).

4 Development of the Lymphatic System

During the last 80 years developmental biology of the vascular system has focused on its cardiovascular part. Our knowledge about the development of the lymphatic system is based almost entirely on studies done at the beginning of the 20th century. The landmark studies of lymphatic development have been done in very diverse species such as pig, frog and chicken, which might explain some of the controversial findings. Despite being the standard model organism, the mouse is still one of the less well characterized species concerning embryonic lymphatic development, although the situation is rapidly changing. Among the nowadays frequently used model organisms only the mouse has a large number of lymph nodes as do humans. However, it is still unknown whether the mouse can serve as a truly good model for all aspects of lymphatic research, due to its small size (creating only a minute amount of hydrostatic pressure), differences in the lymphatico-venous connections and many differences at the molecular level.

In the mouse the development of the lymphatic system starts at E10.5 (corresponding to 6.5-7 weeks of human embryonic development and E4.5 in the chick). By that time the cardiovascular system is already fully functional (Clark and Clark 1920; van der Putte 1975a; b). A discrete population of endothelial cells expressing the lymphatic-specific transcription factor Prox-1 can be already identified at E9.5. They are located on one side of the anterior cardinal vein, and at E10.5 the first lymphatic outgrowths (*lymphatic primordia*) can be identified at that location (Wigle and Oliver 1999; Wigle et al. 2002). It is not understood what induces the outgrowth of these lymphatic primordia. The lymphatic primordia remodel and finally fuse into lymphatic plexuses (*lymph sacs*). There is considerable inter-species variance in the number and exact location of the lymphatic primordia and lymph sacs, although the jugular region seems always to be the main area of lymphatic induction. In mammalian embryos eight lymph sacs have been described: the paired jugular, subclavian and posterior lymph sacs, the unpaired retroperitoneal sac and the cisterna chyli (Sabin 1909; van der Putte 1975a). Two major contradicting theories have emerged about the events that follow the above-mentioned formation of the lymph sacs.

4.1 Centrifugal Sprouting

According to Sabin the peripheral lymphatic system develops from the embryonic lymph sacs exclusively by the sprouting of endothelial cells into the surrounding tissues and organs (Sabin 1902; Clark 1912). Most recent data favors this theory, including expression studies of lymphatic-specific markers (Kaipainen et al. 1995; Kukk et al. 1996) and the Prox-1 knock-out mouse (Wigle et al. 2002). However, there is no agreement among the advocates of the centrifugal sprouting theory about the relationship of the embryonic lymph sacs to the adult lymphatico-venous communications. According to Huntigton and McClure (1910) all lymph sacs lose their connections with the veins and the adult jugular lymphatico-venous communication is a secondary development. Alternatively, the jugular communication of the adult animal is a persisting embryonic communication and data from the mouse argues for this theory (van der Putte 1975b).

4.2 Centripetal Sprouting

McClure and Huntington proposed a vasculogenic mechanism for the establishment of the peripheral lymphatic system. In the mesenchyme lymphatic spaces would arise independently from the veins, fusing into a primitive lymphatic network, which subsequently would spread centripetally and connect to the venous system. The centripetally sprouting lymphatics would either integrate or replace the embryonic lymph sacs (Huntington and McClure 1910; Kampmeier 1912). It is true that the luminal continuum of the lymphatic primordia to the early veins is often not seen (van der Putte 1975a), but a venous origin does not in itself require such, as individual cells might migrate to form the lymphatic primordia.

A model that incorporates both sprouting from lymph sacs and in-situ differentiation of mesenchymal precursors was already proposed in 1932 by van der Jagt and support for this model has been recently gathered. The lymphatics of the avian chorioallantoic membrane (CAM) and perhaps also the wing are apparently not only sprouts from the lymph sacs but also in-situ derivations from mesenchyme. Homotopic grafting of Prox-1 negative, day 2 quail allantoic buds into chick hosts and day 3.5 chick wing buds into quail hosts resulted in lymphatics composed of both donor and host endothelial cells in the graft area (Papoutsi et al. 2001; Schneider et al. 1999).

The development of the lymphatics, just like the one of the blood vessels, is restrained by their evolutionary origin. Much the same as the transient aortic arches in mammalian development are the lymph hearts in birds, which are formed and functional during embryogenesis but disappear by adulthood. In the adult organism lymphatic endothelial cells are normally quiescent, but angiogenic processes – both pathological and physiological – are often accompanied by lymphangiogenesis (Clark and Clark 1932; Ohtani et al. 1998; Paavonen et al. 2000; Mimura et al. 2001).

5 Lymphatics in Different Vertebrates

Until recently there have been misconceptions about the existence of lymphatics in some vertebrate classes. Additionally, sparse data rules out any attempt to draw conclusions about the phylogeny of the lymphatic system; and almost all of the research on comparative anatomy was done during the 19th and early 20th century with the limitations of that period.

5.1 Fish

Already in 1919 Mayer tried to explain the contradictory findings about the piscine lymphatics by the existence of a secondary vascular system. However, only in 1981 could

Vogel and Claviez experimentally prove this existence. The secondary vascular system constitutes a separate, parallel circulatory system and includes the vessels earlier assumed to be lymphatics (Hoyer 1934). It starts from the systemic arteries, forms its own capillary networks, which supply mainly the oral mucous membranes and the skin, and returns to the systemic venous system. It functions presumably in skin respiration, osmoregulation and immune defence. Based on its anatomical and functional characteristics it has been hypothesized that the secondary circulation might be an evolutionary predecessor of the lymphatic system (Steffensen and Lomholt 1992). There is evidence for a "true" lymphatic system in lungfish and it is thus reasonable to speculate that the first occurrence of a lymphatic system was associated with the transition from aquatic to terrestrial life (Laurent et al. 1978).

Growth factors and receptors that regulate lymphatic growth and development in higher vertebrates are present in fish, but their relevance for the secondary vascular system has not been analyzed (Stainier et al. 1995).

Molecules of the same growth factor family have also been identified in invertebrates, that lack endothelial cells altogether. In Drosophila these molecules direct embryonic blood cell migration. Probably only recently did these molecules assume their roles in blood vessel and lymphatic development, and it is conceivable that blood vessels evolved from blood cells (Duchek et al. 2001; Heino et al. 2001; Cho et al. 2002). In ontogenesis, however, the inverse can be observed (Ciau-Uitz et al. 2000; de Bruijn et al. 2000).

5.2 Amphibians and Reptiles

All amphibian orders are believed to have a lymphatic system. It is comparable to the mammalian system except in frogs and toads, where the superficial initial lymphatics fuse during metamorphosis to form cutaneous lymph sacs in the adult animal (Hoyer 1934; Kotani 1990). Characteristic for all amphibians are the lymph hearts, which are located at the entry points of lymph into the veins. Entry points of the lymph into the blood circulation can occur in vertebrates in three different areas: in the jugular, the lumbar and the caudal region. Most amphibian lymphatic systems communicate with the venous system in all three areas. Lymph hearts range in number from four to six in frogs to over two hundred in caecilians. Unlike their name suggests, the main function of lymph hearts is probably not the propulsion of the lymph, but rather maintaining the directionality of lymphatic flow and regulating the entry of lymph fluid into the circulation. In reptiles lymphatico-venous communications exist in the caudal and the jugular regions, but lymph hearts are found only in the caudal region (Hoyer 1934).

5.3 Birds and Mammals

Mammals and most birds do not possess lymph hearts. However, unlike other vertebrates, mammals and aquatic birds possess lymph nodes. Vertebrates differ significantly in the number of lymph nodes: in humans there are between 400 and 500 lymph nodes while ducks have only four of them (Weidenreich et al. 1934). In most mammals multiple lymphatico-venous communications are formed during development. Usually only the paired communication in the jugular region persists into adulthood. Several mammalian species maintain lumbar communications into the inferior caval vein and the renal vein, draining the lymphatics of the lower extremities and the mesentery (Silvester 1912; Job 1918).

There is substantial intra-species variability in the setup of the lymphatic system. Humans have usually a paired jugular communication, but additional lymphatico-venous communications at both central and peripheral locations are not uncommon (Wolfel 1965;

Threefoot and Kossover 1966; Pressman et al. 1967; Aboul-Enein et al. 1984).

It is not known whether the need for drainage was the primary selection force for the development of the lymphatics. Apart from a high-pressure cardiovascular system other evolutionary triggers might have been involved. Two of them, the hydrostatic pressure associated with the transition of aquatic to terrestrial life and the transition from poikilothermic to homeothermic temperature regulation, are associated with an increase in blood pressure, while a third one, the development of an adaptive immune system, does not require a high-pressure cardiovascular system per se.

6 Pathology

6.1 Blood Vessels and Lymphatic Vessels in Disease

Angiogenesis or the lack thereof is a key event in the development and progression of major pathological conditions including diabetic retinopathy, psoriasis, rheumatoid arthritis, cardiovascular diseases, and tumor growth (reviewed by Carmeliet and Jain 2000). Much of the interest in angiogenesis research is driven by the idea of therapeutic intervention. Pro-angiogenic proteins like VEGF and VEGF-C have been used in clinical studies to boost the growth and perfusion of blood vessels after vascular injuries like myocardial infarction (Isner et al. 1996; Witzenbichler et al. 1998). Also, because of convincing evidence that solid tumors are angiogenesis-dependent, anti-angiogenic compounds have entered clinical trials (Hanahan and Folkman 1996). Similar to developing embryos, tumors can only grow beyond the limits of diffusion by establishing a vascular network for the distribution of oxygen and nutrients. An imbalance between pro-angiogenic and anti-angiogenic factors results in vessel ingrowth and is followed by rapid tumor expansion (Hanahan and Folkman 1996). Despite encouraging results obtained in several mouse models of anti-angiogenic tumor therapy and pro-angiogenic therapy of ischemia, success in preclinical and clinical trials is limited (Cao 2001; Hammond and McKirnan 2001). Caution has been recommended (Carmeliet 2000b) and also principal objections against the safety and feasibility of such therapies have been raised (Blagosklonny 2001). In developed countries the angiogenesis-related cardiovascular and neoplastic diseases are major health issues, whereas lymphatic disorders are relatively rare.

6.2 Lymphedema

Insufficiency of lymphatic transport can result in lymphedema, which can either be hereditary or with unknown etiology (*primary lymphedema*), or a consequence of a previous disease or trauma (*secondary* or *acquired lymphedema*). Iatrogenic lymphedema, and especially postmastectomy edema, represents probably the most common lymphatic condition in civilized countries. Its incidence has been estimated to between 6 to 30% after surgical treatment of mammary carcinoma (Petrek and Heelan 1998). Surgical damage to the lymphatics is commonly thought to be the cause of post-surgical edema, but also other reasons such as compromised venous return seem to be involved (reviewed by Pain and Purushotham 2000). The worldwide most common cause of lymphedema is, however, filariasis – mostly caused by infection with *Wuchereria bancrofti* or *Brugia malayi*. These parasitic nematodes are transmitted by mosquito bites. The parasite lives and reproduces in the lymphatic system causing a massive lymphatic dilatation in early stages of the disease. In in advanced disease, lymphatic transport is blocked leading to an extreme enlargement of the limbs or other areas of the body called *elephantiasis* (Rao et al. 1996; Dreyer et al. 2000).

In hereditary lymphedema lymphatic vessels can either be hypoplastic or hyperplastic, but non-functional. In addition to some broad-spectrum syndromes such as Ulrich-Turner and Noonan, that are associated with lymphedema, a large number of distinct lymphedema syndromes has been described. The current phenotypic classification seems inadequate, based on recent clinical and genetic data showing that the same genetic cause can give rise to several distinct phenotypes, and the same phenotype can be caused by distinct genetic alterations (Kääriäinen 1984; Ferrell et al. 1998; Finegold et al. 2001).

6.2.1 Hereditary Lymphedema Type I and II

Type I hereditary lymphedema (*Milroy disease*, OMIM 153100) is an early onset form of hereditary lymphedema. In these patients, the initial superficial lymphatics of edematous areas cannot be demonstrated by fluorescence microlymphography and are believed to be absent or highly hypoplastic. However, in non-edematous areas superficial lymphatics are present (Bollinger et al. 1983).

In some families inheritance is strongly linked to dominant missense mutations in the VEGFR-3 gene on chromosome 5 (Karkkainen et al. 2000). However, penetrance is incomplete or variable in these families. Other families showed additional linkage to multiple loci on chromosomes 3, 11 and 18, unrelated to any known target genes. Thus, oligogenic pattern of inheritance, modifier genes and environmental factors might be necessary to explain the hereditary patterns. Type II hereditary lymphedema (*Meige disease*, OMIM 153200) differs from type I by a later disease onset (around puberty), and its etiology appears even more complex with only 10% of the families showing dominant pattern of inheritance and a penetrance of 40% (Holberg et al. 2001).

6.2.2 Lymphedema-Distichiasis Syndrome

Dominant mutations in the transcription factor FOXC2 have been identified as the cause of lymphedema-distichiasis syndrome (OMIM 153400; Fang et al. 2000; Finegold et al. 2001). In addition to lymphedema distichiasis three other lymphedema syndromes co-segregated with FOXC2 mutations: type II hereditary lymphedema, lymphedema/ptosis (OMIM 153000) and lymphedema/yellow-nail syndrome (OMIM 153300). All indentified FOXC2 mutations result in a truncated protein and the observed phenotype is likely a result of haploinsufficiency.

6.2.3 Lymphangiectasia

Lymphangiectasia is a lymphatic disorder characterized by dilated dysfunctional lymphatics. The condition can be limited to a specific organ. The lungs are affected in hereditary pulmonary cystic lymphangiectasia (OMIM 265300) and the intestine in Hennekam lymphangiectasia-lymphedema syndrome (OMIM 235510; Hennekam et al. 1989; Gilewski et al. 1996). The causes of lymphangiectasia and lymphedema are thought to be similar and both conditions do occur jointly in syndromes such as Noonan Type I (OMIM 163950), Hennekam or hereditary intestinal lymphangiectasia (OMIM 152800). Similar to lymphedema, also acquired forms are known (Celis et al. 1999).

6.3 Lymphatic Neoplasms

Occasionally, neoplasms are derived from lymphatic endothelial cells. Lymphangiomas constitute approximately 5% of all benign lesions of infancy and childhood (Zadvinskis et al. 1992). Since lymphangiomas can present either as localized mass or as a diffuse tumor, it is questionable whether all lymphangiomas represent true neoplasms (Scalzetti et al. 1991). Unlike lymphangioma, lymphangiosarcoma is a true malignant lesion of lym-

phatic endothelial cells. Mostly it occurs as a complication of post-mastectomy edema (*Stewart Treves syndrome*; Janse et al. 1995).

6.4 Tumor Lymphangiogenesis

The lymphatic system serves as the primary pathway for metastatic spread of tumor cells to regional lymph nodes, and possibly also to distant organs. The prognostic value of lymph node metastasis was recognized long before the concept of lymphangiogenesis both within and adjacent to tumors became widely accepted (Fisher et al. 1969; Carter et al. 1989). Tumor lymphangiogenesis occurs in experimental models in mice with important implications for metastasis (Karpanen et al. 2001; Mandriota et al. 2001; Skobe et al. 2001; Stacker et al. 2001). It is, nevertheless, still unclear to what extent lymphangiogenesis occurs in human cancers, and what the consequences are for cell dissemination (Leu et al. 2000; Birner et al. 2001; Jackson et al. 2001; Schoppmann et al. 2001).

6.5 Therapeutic Lymphangiogenesis

The regeneration of lymphatic vessels was observed by Clark and Clark already in 1932. However, expertise in therapeutic lymphangiogenesis is just emerging (Karkkainen et al. 2001). Several recent papers have been shown that in angiogenic therapy the balance between harm and help is not trivial (Masaki et al. 2002) and the use of single molecules is likely to be insufficient (Richardson et al. 2001). Influencing the molecular decision makers such as Hypoxia-inducible factor-1 (HIF-1) instead of the effector molecules such as VEGF might be easier than the futile attempt to mimic the temporally and spatially complex growth factor cocktail of mother nature (Vincent et al. 2000; Elson et al. 2001). Whichever pro-angiogenic therapy, it is possible that pro-angiogenic or pro-lymphangiogenic therapy leads to accelerated cancer progression and metastasis.

So far no good animal model has been developed for aquired lymphedema, but a mouse model of hereditary lymphedema does exist: the *Chy* mouse. *Chy* mice carry a mutant Vegfr-3 allel coding for a kinase-dead receptor. In this model VEGF-C was used to compensate for insufficient VEGFR-3 signaling. This resulted in the growth of new functional lymphatics (Karkkainen et al. 2001). It is not clear why VEGF-C does induce lymphatic sprouting in these mice, but fails to do so in the keratin-14 VEGF-C transgenic mice. The VEGF-C transgenic mice were edematous, indicating that also too much VEGFR-3 signaling can impair lymphatic function (I). From the visceral lymphatics in the Chy mouse, only the lacteals are aplastic although the VEGFR-3 mutation is assumed to be dominant negative in all lymphatic endothelial cells and there is circumstantial evidence that lymphangiogenesis might occur independently of VEGFR-3 activation (Taija Makinen, personal communication). Thus many molecular players are still to be identified. In conclusion, therapeutic modulation of vascular growth in pathologic conditions represents the major challenge in the fields of both angiogenesis and lymphangiogenesis.

7 VEGFs

The last 20 years of angiogenesis research have been dominated by molecular biology. The identification of the molecular players started with the discovery of VEGF in 1989 (Keck et al. 1989; Leung et al. 1989; Plouët et al. 1989) and the discovery of VEGF-C in 1996 (Joukov et al. 1996; Lee et al. 1996). Several large scale sequencing projects have contributed to the identification of genes involved in angiogenesis, and the focus of today's research is to understand rather than to discover. The task of understanding seems

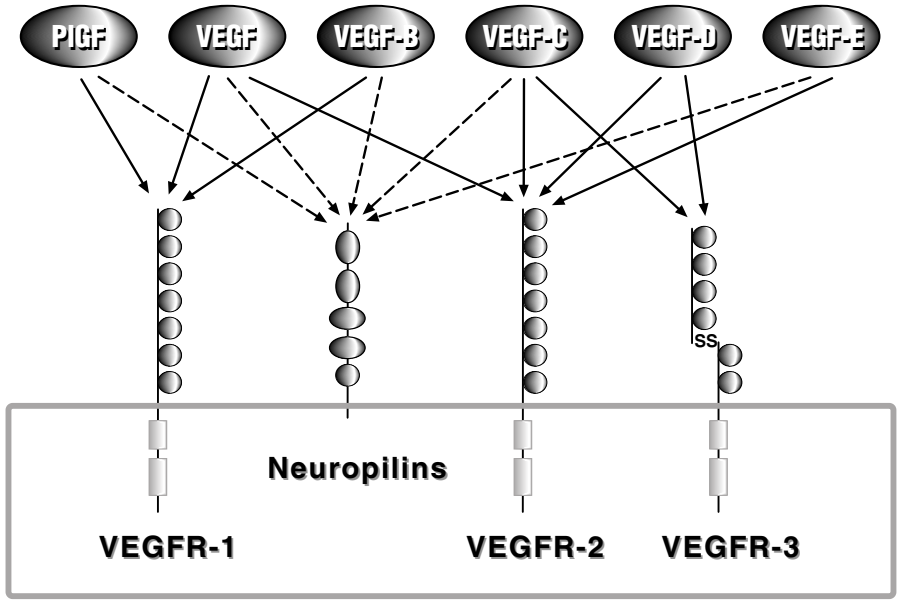

Figure 2. VEGF family members and their receptors

tremendous, despite major technological advances such as genomics and proteomics.

Polypeptide growth factors and their receptors are major components of the regulatory machinery governing angiogenesis. Central positions in this machinery are occupied by the VEGF receptor tyrosine kinases. VEGF receptors are largely specific for endothelial cells. In mammals three VEGF receptors interact with five different VEGFs, each of which has a characteristic receptor-binding pattern (Figure 2). However, other growth factor and receptor families provide major contributions to vessel differentiation, notably the Tie receptors with their angiopoietin ligands (reviewed by Jones et al. 2001), the ephrins and their receptors (reviewed by Cheng et al. 2002), the neuropilins, which can act as co-receptors for several VEGF family members (reviewed by Neufeld et al. 2002) and the PDGFs, which do not act directly on endothelial cells, but instead recruit pericytes and smooth muscle cells to coat the endothelial tube, which is indispensible for vessel stabilization (reviewed by Betsholtz et al. 2001).

7.1 VEGF - Master Effector of Angiogenesis

The "par excellence" key molecule of vascular development is vascular endothelial growth factor (VEGF, also called VEGF-A to distinguish it from the other VEGF family members). The function of VEGF in vasculogenesis and angiogenesis has been extensively analyzed and reviewed (e.g. Neufeld et al. 1999; Ferrara 2001). VEGF binds to VEGFR-1 and VEGFR-2. VEGFR-2 seems to mediate most, if not all, of the biological effects of VEGF, while VEGFR-1 has probably a modifying function, mostly by acting as a decoy receptor. In VEGFR-2 knockout mice both hematopoiesis and vasculogenesis are blocked (Shalaby et al. 1995), whereas in VEGFR-1 knockout mice the remodeling of the primary vascular plexus fails due to increased hemangioblast commitment of mesenchymal precursors (Fong et al. 1995; Fong et al. 1999). Mice in which the kinase domain

of VEGFR-1 is deleted appeared normal, arguing strongly for a primarily non-signaling function of VEGFR-1 (Hiratsuka et al. 1998). Additionally, the viral VEGF homologue VEGF-E, which does not bind VEGFR-1, is able to carry out most of the functions that VEGF does (Meyer et al. 1999; Wise et al. 1999). In mouse development, VEGF expression starts at E 4.0 (Miquerol et al. 1999) before the onset of vasculogenesis. Activation of VEGFR-2 by VEGF has been both implicated in the migration (Shalaby et al. 1997) and the induction (Bautch et al. 2000) of the endothelial precursor cells. Targeted inactivation of even a single VEGF allele results in embryonic lethality, indicating that tightly regulated VEGF levels are a prerequisite for embryonic development (Carmeliet et al. 1996; Ferrara et al. 1996).

VEGF is induced by hypoxia, and although large vessels are genetically determined, at least the vascularization of some embryonic tissues seems to be directed by hypoxia (Stone et al. 1995). In addition to proliferation, VEGF stimulates endothelial cell migration and vessel permeability (Dvorak et al. 1995). Although endothelial cells are the primary target, VEGF also elicits responses in cells of the monocyte/macrophage hematopoietic lineage, including monocytes (Clauss et al. 1990; Barleon et al. 1996), dendritic cells (Gabrilovich et al. 1996) and osteoclasts (Midy and Plouet 1994; Gerber et al. 1999).

In addition to the predominant $VEGF_{165}$ species, five other isoforms are generated in humans by alternative splicing: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{183}$, $VEGF_{189}$, and $VEGF_{206}$ (Leung et al. 1989; Houck et al. 1991; Tischer et al. 1991; Poltorak et al. 1997; Lei et al. 1998). The isoforms vary in their expression pattern and their binding properties for heparan sulfate proteoglycans, extracellular matrix and co-receptors neuropilin-1 and -2. $VEGF_{121}$ binds neither heparin nor neuropilin, while $VEGF_{145}$ and $VEGF_{165}$ do (Soker et al. 1996; Gluzman-Poltorak et al. 2000). The interaction with neuropilins might explain the differential biological potency, despite having an equal affinity towards VEGF receptors. For cultured endothelial cells $VEGF_{121}$ is substantially less mitogenic than $VEGF_{165}$ (Keyt et al. 1996a), and in new-born gene targeted mice $VEGF_{120}$ cannot compensate for the loss of the longer isoforms, leading to ischemic cardiomyopathy and death (Carmeliet et al. 1999b). The ability to interact with glycosaminoglycans also prolongs the biological half-life of $VEGF_{165}$ compared to $VEGF_{121}$, since glypican-1 can act as an extracellular "chaperone" for VEGF (Gengrinovitch et al. 1999).

The structure of VEGF

VEGF and PDGFs are members of the cystine knot growth factor superfamily (McDonald and Hendrickson 1993). PDGF-B was the first member of the PDGF/VEGF family whose structure was solved by X-ray crystallography, followed by VEGF (both alone and complexed to domain 2 of VEGFR-1; Oefner et al. 1992; Muller et al. 1997b; Wiesmann et al. 1997) and PlGF (Iyer et al. 2001). These structures describe the receptor-binding domain, which is largely identical with the VEGF homology domain. Besides the VEGF homology domain, most PDGF/VEGF family members contain at least one additional domain with unique characteristics. In case of VEGF this additional domain can fold independently from the VEGF homology domain, and its structure was determined by NMR spectroscopy (Fairbrother et al. 1998).

VEGF is an antiparallel dimer, covalently linked by two disulfide bridges between Cys-51 and Cys-60. Its cystine knot is located lateral to a central 4-stranded antiparallel β-sheet. One VEGF molecule ligates two receptor molecules by virtue of its two receptor-binding sites, which lie at both ends of the flat and elongated molecule (see Figure 4A). Both subunits contribute to each individual binding

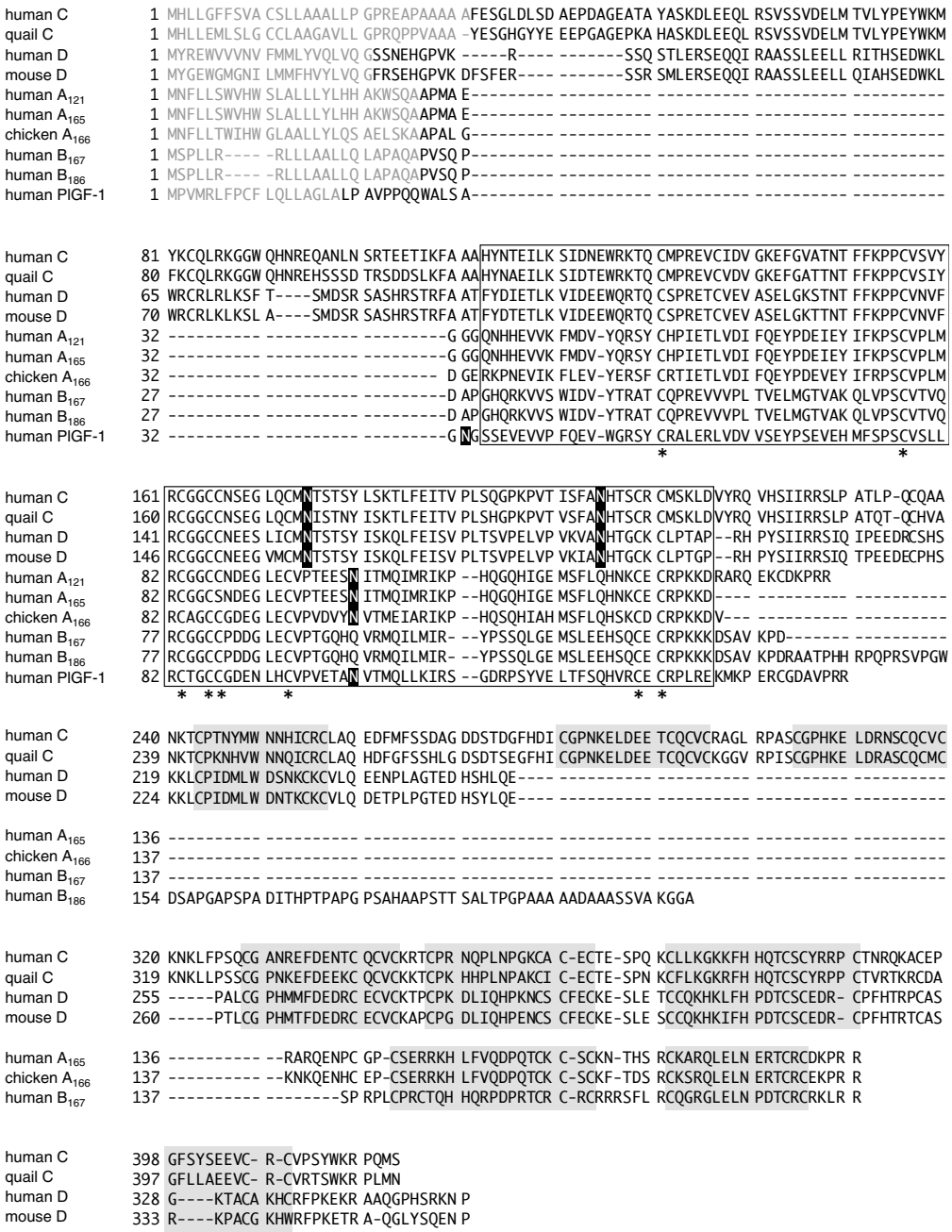

Figure 3. Alignment of selected VEGFs

GREY CHARACTERS – signal peptide, GREY BACKGROUND – BR3P motif, BLACK BACKGROUND – putative N-glycosylation sites; the VHD is boxed and the 8 conserved cysteines are indicated by asterisks (∗).

site in a 2 to 1 surface area ratio (Muller et al. 1997a; Muller et al. 1997b; Wiesmann et al. 1997). There is no conclusive data about the necessity of disulfide bridges for structural integrity and thermodynamic stability of cystine-knot growth factors. Dimerization of VEGF-C, VEGF-D and NGF does not utilize intersubunit disulfide bridges (McDonald et al. 1991; Joukov et al. 1996). Intersubunit disulfide bridges are dispensable for the integrity of PDGF-BB (Kenney et al. 1994). The integrity of VEGF produced by mammalian cells was compromised when the intersubunit bridge-forming cysteines were mutated (Potgens et al. 1994). However, VEGF mutants produced in E.coli, that lacked either inter- or intrasubunit cysteine bridges could be folded in vitro, but these mutants were not tested for their biological activity (Heiring and Muller 2001).

Both $VEGF_{121}$ and VEGF-C contain an additional cysteine residue in or close to the VEGF homology domain, which in case of $VEGF_{121}$ has been shown to be capable of forming an additional interchain disulfide bond (Keck et al. 1997).

Receptor domains involved in VEGF binding

Human VEGF receptors are glycosylated type I integral membrane proteins. Seven immunoglobulin-like domains form the extracellular portion and a split kinase domain forms most of its intracellular part. VEGF receptors are close relatives of the PDGF receptors.

Domains 6 and 7 of VEGF receptors are thought to be a result of a duplication event in a putative common ancestor gene of the VEGF receptors, PDGF receptors, the CSF-1 receptor (c-Fms) and the SCF receptor (c-Kit). Thus immunoglobulin-like domains 1 to 5 of VEGF receptors correspond to the whole extracellular domain of PDGF receptors, c-Fms and c-Kit (Claesson-Welsh et al. 1988, 1989; Shibuya et al. 1990).

The second Ig-like domain of VEGFR-1 ($VEGFR\text{-}1_{D2}$) was shown to be sufficient for VEGF binding (Wiesmann et al. 1997), contradicting earlier studies (Davis-Smyth et al. 1996; Barleon et al. 1997; Cunningham et al. 1997b). The crystal structure rationalized this discrepancy, showing that earlier domain deletions cut into the last β-strand of domain 2 or had extra residues that were likely to destabilize the overall folding (Wiesmann et al. 1997). Deletion constructs containing only domains 2 and 3 of both VEGFR-1 and VEGFR-2 bind VEGF with nearly wild type affinities. In VEGFR-1 further deletion of domain 3 decreases the affinity for VEGF only 20-fold, while the corresponding deletion in VEGFR-2 results in a 1000-fold decrease, showing that the relative importance of domain 3 differs between VEGFR-1 and VEGFR-2 (Wiesmann et al. 1997; Fuh et al. 1998).

Despite several non-canonical features $VEGFR\text{-}1_{D2}$ is a member of the I-set of the immunoglobulin superfamily (Harpaz and Chothia 1994; Wiesmann et al. 1997). The sequence deviations in $VEGFR\text{-}1_{D2}$ from I-set positions are conserved in the second domain of other closely related receptors such as VEGFR-2, VEGFR-3, PDGFRα, PDGFRβ, c-fms and c-kit (Wiesmann et al. 1997). These related receptors also bind their ligand (at least partly) with their second domains (Blechman et al. 1995; Mahadevan et al. 1995; Lemmon et al. 1997; Fuh et al. 1998). Similarly to FGFs, heparin or heparan sulfate proteoglycans have been shown to be essential for high-affinity binding of VEGF to VEGFR-2 (Gitay-Goren et al. 1992). In VEGFR-2 the heparin-binding domain does not map anywhere near the ligand-binding domain (Dougher et al. 1997), while in the FGF2/FGF receptor-1/heparin complex two heparin molecules are integral components of the receptor-ligand interface (Schlessinger et al. 2000).

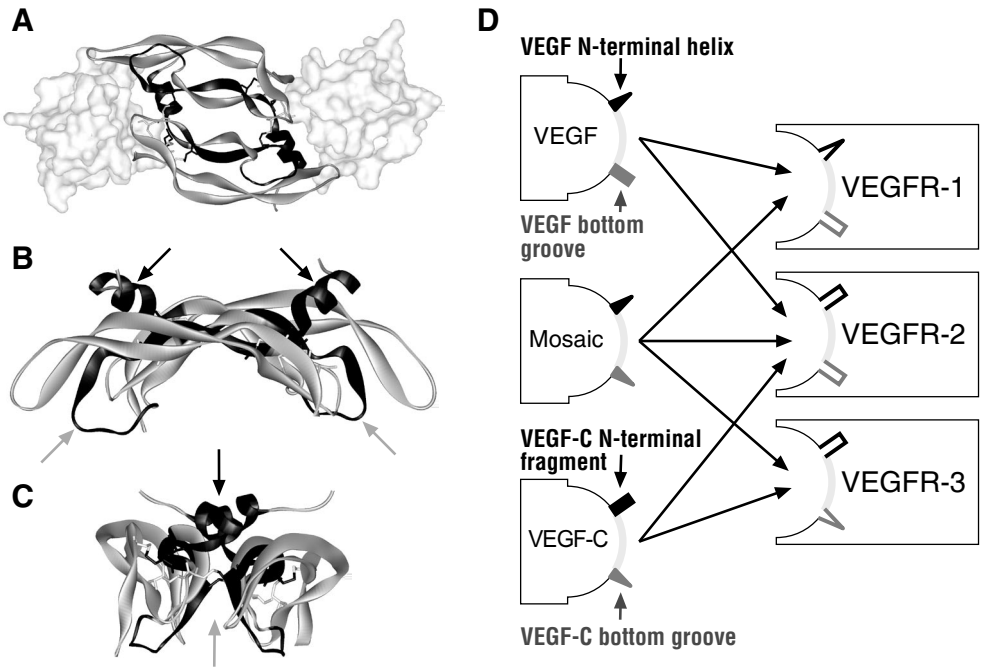

Figure 4. Structure of VEGFs: Uncoupling receptor binding and specificity

The location of the specificity-determining regions of VEGF and VEGF-C shown at a model of VEGF. **A** VEGF complexed to domain 2 of VEGFR-1 (bottom view). **B** and **C** VEGF (side views). The N-terminal fragment of VEGF-C (black arrow) prevents interaction with VEGFR-1 and corresponds to the N-terminal α-helix of VEGF. The bottom groove of VEGF (grey arrow) prevents interaction with VEGFR-3; light gray ribbon rendering – VEGF, white space-filling rendering – domain 2 of VEGFR-1, black ribbon rendering – major specificity-determining elements. **D** Schematical illustration of the major specificity-determining elements; black – N-terminal fragment, dark gray – bottom groove; light gray – promiscuous receptor binding interface.

VEGFR-1 binding determinants

Mutagenesis of VEGF had suggested three negatively charged key residues for the interaction with VEGFR-1 (Asp-63, Glu-64 and Glu-67; Keyt et al. 1996b), but the co-crystallization of VEGF with VEGFR-1_{D2} showed, that only Asp-63 participated in receptor binding via a charge-mediated interaction with Arg-224 of VEGFR-1_{D2}. The VEGFR-1 binding interface is dominated by hydrophobic contacts and is surprisingly flat, showing no predominant knob-into-hole interactions (Wiesmann et al. 1997). VEGFR-1_{D2} does not undergo any significant conformational changes upon ligand binding (Starovasnik et al. 1999), but the flexible loops that surround the rigid core of VEGF seem to be necessary for achieving promiscuity in binding two different receptors (Muller et al. 1997a).

VEGFR-2 binding determinants

The VEGFR-2 binding interface of VEGF was identified by alanine-scanning mutagenesis (Muller et al. 1997b). Seven mostly hydrophobic residues were found to be significant for strong VEGFR-2 binding, five of which are localized in the VEGFR-1 binding interface of VEGF (Phe-17, Ile-46, Gln-79, Ile-83 and Pro-85), suggesting that the binding sites

for VEGFR-1 and VEGFR-2 are very similar. The two remaining residues are located outside the VEGFR-1_{D2} binding interface (Ile-43 and Glu-64), but may be part of the binding interface in the native complex (Wiesmann et al. 1997). Previous studies had identified Arg-82, Lys-84 and His-86 as the most important residues for KDR binding (Keyt et al. 1996b), however this mutagenesis study was not systematic and did not include 6 of the 7 amino acids that Muller et al. identified later in a systematic approach (Muller et al. 1997b; Wiesmann et al. 1997).

Monomeric versus dimeric binding

Predimerized VEGFR-2 binds VEGF about 100-fold more tightly than monomeric VEGFR-2, while predimerized VEGFR-1 binds VEGF only 2-fold more tightly than monomeric VEGFR-1 (Wiesmann et al. 1997; Fuh et al. 1998). Receptor–receptor interactions might be more prominent in VEGFR-1 than in VEGFR-2, especially under the assay conditions that did not include heparin, which is necessary for the receptor-receptor interaction of VEGFR-2 (Dougher et al. 1997).

The heparin binding domain of VEGF

The C-terminal domain of $VEGF_{165}$ (also called $VEGF_{55}$) constitutes a heparin-binding domain similar to the one of plasminogen, but unrelated to the FGF type heparin binding domain (Fairbrother et al. 1998). Based on the homology of the VEGF heparin-binding domain to the C-terminal tail of VEGF-C and -D, one could expect VEGF-C to bind heparin, although such binding has not been reported in the literature.

7.2 PlGF, VEGF-B and Other Problem Children

Placenta growth factor (PlGF) and VEGF-B differ from VEGF in that they do not bind VEGFR-2 (Park et al. 1994; Olofsson et al. 1998). Both PlGF and VEGF-B exist in two different isoforms (PlGF-1 and PlGF-2; VEGF-B_{167} and VEGF-B_{186}), which are generated by differential mRNA splicing (Maglione et al. 1993; Olofsson et al. 1996). While alternative splicing of VEGF and PlGF mRNA successively integrates additional in-frame basic stretches into the polypeptide, the alternative splicing of VEGF-B mRNA results in a frame shift and two non-homologous C-termini. The C-terminus of VEGF-B_{167} is similar to $VEGF_{55}$, whereas VEGF-B_{186} has a unique hydrophobic C-terminus, which can be for the most part removed by proteolytic processing at Arg-127. This cleavage regulates the affinity of VEGF-B_{186} for its co-receptor neuropilin-1, which also binds VEGF-B_{167} and PlGF-2 (Migdal et al. 1998; Makinen et al. 1999). Unlike all other VEGF family members, VEGF-B does not contain any N-linked carbohydrates: VEGF-B_{167} is not glycosylated at all and VEGF-B_{186} becomes O-glycosylated (Olofsson et al. 1996).

VEGF-B has a wide tissue distribution that overlaps substantially with that of VEGF. In adults it is most abundant in heart, skeletal muscle and brown fat. VEGF-B_{167} seems to be the predominant isoform under physiological conditions (Olofsson et al. 1996; Lagercrantz et al. 1998; Aase et al. 1999; Li et al. 2001). The function of PlGF and VEGF-B needs still further characterization. In most systems VEGFR-1 mediated biological responses are absent or weak (II; Waltenberger et al. 1994; Sawano et al. 1996). Both PlGF and VEGF-B knockout mice are viable and fertile, and develop a normal vasculature. Studies in these knockout mice link PlGF specifically with repair angiogenesis (Carmeliet et al. 2001; Luttun et al. 2002) and VEGF-B weakly with cardiac function (Aase et al. 2001).

Some predators make use of VEGF-like molecules with devastating results for their prey: molecules with approximately 50% identity to VEGF are apparently common components of snake venoms. In addition to the induction

of hypotensic shock, the increase in vascular permeability presumably facilitates access of the neurotoxic venom components to their target cells (Komori et al. 1999; Gasmi et al. 2000; Junqueira de Azevedo et al. 2001; Gasmi et al. 2002). Another innovative use for VEGF-like molecules can be seen in some parapoxviruses. *Orf virus* strains NZ2, NZ7 and D1701 have captured a VEGF-like gene from their mammalian hosts, and the vascular lesions caused by these viral VEGFs (collectively known as *VEGF-E*) are probably instrumental in viral spread (Lyttle et al. 1994; Ogawa et al. 1998; Meyer et al. 1999; Wise et al. 1999; Savory et al. 2000).

7.3 The Lymphangiogenic Factors: VEGF-C and VEGF-D

A close paralogue of VEGFR-1 and –2 is VEGFR-3. Its expression becomes restricted to lymphatic endothelial cells during embryogenesis (Kaipainen et al. 1995; Kukk et al. 1996). The search for its ligand led to the identification two lymphangiogenic growth factors: VEGF-C and VEGF-D (Joukov et al. 1996; III).

VEGF-C and VEGF-D specifically induce the proliferation of lymphatic endothelial cells in transgenic mice and in the CAM (I; II; Veikkola et al. 2001; own unpublished data). In the same assays VEGF is specific for blood vessels and does not induce any changes in the lymphatic vasculature (II; Detmar et al. 1998). However, several reports confirm that both VEGF-C and VEGF-D can exercise significant angiogenic potency both in vitro and in vivo (Lee et al. 1996; Cao et al. 1998; Witzenbichler et al. 1998; Marconcini et al. 1999; Byzova et al. 2002). Discrepancies concerning the angiogenic versus the lymphangiogenic potential of VEGF-C and VEGF-D are believed to be a result of different extracellular processing (Joukov et al. 1997; Stacker et al. 1999a). This, however, does not explain experimental results showing that also the lymphangiogenic potential of mature VEGF-C exceeds by far its angiogenic potential (II).

Mice deficient in VEGFR-3 died from cardiovascular failure at E9.5 (Dumont et al. 1998) due to an impaired remodeling and maturation of large vessels. VEGFR-3 thus appears to a have an essential role before the formation of the lymphatic system, when VEGFR-3 is still ubiquitously expressed on all endothelial cells. In VEGF knock-out embryos endothelial differentiation is not completely blocked unlike in the VEGFR-2 knock-out mice, suggesting that VEGF-C indeed does activate VEGFR-2 in early embryonic development (Carmeliet et al. 1996; Ferrara et al. 1996; Shalaby et al. 1997). At least in vitro VEGF-C was able to replace VEGF in a differentiation assay using avian hemangioblasts (Eichmann et al. 1997). More insight into the role of VEGFR-3 in lymphatic development could be obtained by a conditional knockout. However, caution was recommended when interpreting mouse VEGFR-3 data, since in humans an endogenous retrovirus disrupts the splice pattern, giving rise to a C-terminally shortened receptor isoform, whose signaling properties differ from the long isoform (Pajusola et al. 1993; Borg et al. 1995; Hughes 2001).

7.3.1 Molecular Features of VEGF-C and VEGF-D

VEGF-C and VEGF-D differ from other VEGF family members by the presence of long N- and C-terminal extensions flanking the VEGF-homology domain (Joukov et al. 1996; Lee et al. 1996; Orlandini et al. 1996; Yamada et al. 1997; III). The N-terminal propeptide does not contain any recognizable motifs, while the C-terminal domain contains a repetitive cysteine pattern (Cys-X_{10}-Cys-X-Cys-X-Cys), homologous to a motif first identified in a silk-like secretory Balbiani ring 3 protein produced by larval salivary glands of the midge *Chironomus tentans.*

The VEGF homology domain (VHD) of VEGF-C exhibits 35% identity to VEGF. It is encoded by exons 3 and 4 of the seven exons (Chilov et al. 1997), which is a feature conserved in other members of the VEGF family (Tischer et al. 1991; Maglione et al. 1993; Olofsson et al. 1996). The VEGF homology domains of VEGF-C and VEGF-D are 60% identical. VEGF-C is synthesized as a precursor protein, which undergoes subsequent proteolytic processing (Joukov et al. 1996; Joukov et al. 1997). The C-terminal domain is cleaved upon secretion, but remains bound to the N-terminal domain by disulfide bonds, giving rise to a disulfide linked tetramer composed of 29-kDa and 31-kDa polypeptides. Proteolytic processing of the N-terminal propeptide releases the mature form, which consists of two 21-kDa polypeptide chains corresponding largely to the VEGF homology domain (Joukov et al. 1997). The 29/31-kDa form seems to be the most prevalent form of VEGF-C in various biological systems (Lee et al. 1996; Hu et al. 1997; Joukov et al. 1997; Eichmann et al. 1998; Hiltunen et al. 2000). Incomplete and additional proteolytic processing leads to two minor fragments that migrate on reducing SDS-PAGE with an apparent molecular weight of 15 and 43 kDa. The 15-kDa fragment represents the N-terminal propeptide while the 43-kDa form presumably represents its complement (the VHD and the C-terminal domain). VEGF-D is processed in a similar fashion (Stacker et al. 1999a).

Both the full-length and the mature forms of human VEGF-C bind VEGFR-3 with high affinity (Joukov et al. 1997), while high affinity binding to VEGFR-2 requires proteolytic processing. Full length VEGF-C and the 29/31-kDa form bind VEGFR-2 weakly, while the mature form and the 43-kDa form bind efficiently (Joukov et al. 1997; own unpublished data). The receptor binding profile of VEGF-C seems to be conserved among species, but not the one of VEGF-D, as mouse VEGF-D does not bind mouse VEGFR-2 (Baldwin et al. 2001a). Similar to VEGF, glycosylation of VEGF-C is no prerequisite for receptor binding, but is necessary for efficient secretion (own unpublished data).

7.3.2 Structure of VEGFR-3 and Its Ligands

Neither the structure of VEGFR-3 nor of any of its ligands has been solved. The high homology of VEGF-C and VEGF in the VHD allows for molecular modeling of this domain and together with mutational data offers some limited insights into the nature of the interaction (IV). The primary sequences of VEGF-C and VEGF show major differences in two regions which code for the N-terminal part and for the flexible loop between β-strands 5 and 6. The N-terminal part of the VHD forms an α-helix in both VEGF and PlGF (Muller et al. 1997b; Iyer et al. 2001), but is disordered in PDGF-BB (Oefner et al. 1992) and probably also in VEGF-C. Mutational analysis shows that these two regions are involved in determining receptor specificity in VEGFs (IV). VEGF-C loses its ability to bind and activate VEGFR-2 when Cys-156 is mutated into serine (Joukov et al. 1998). In other members of the PDGF/VEGF family, cysteine residues homologous to this Cys-156 are involved in interchain disulfide bonding, whereas VEGF-C and VEGF-D are noncovalent dimers (Joukov et al. 1997; Stacker et al. 1999a). This is additional evidence for a role of the bottom groove in receptor binding as previously proposed for VEGF and VEGFR-1 (Wiesmann et al. 1997). Indeed, residues of this region are indispensable for VEGFR-3 binding (IV). Similarly to VEGFR-1 binding, VEGFR-3 binding does not require the presence of the third domain of the receptor (IV). Surprisingly enough, the first domain appeared necessary, although it is unlikely that domain 1 interacts directly with VEGF-C (IV).

The existence of mosaic VEGFs that bind all VEGF receptors adds prove to the idea that the binding of VEGFs to their receptors uses a very similar mechanism. The inability of a VEGF to bind to a certain VEGF receptor can be traced to limited specific structural elements (IV). This partial uncoupling of receptor binding determinants from receptor specificity determinants is reminiscent of human growth hormone (HGH) and prolactin. HGH can bind both the HGH receptor and the prolactin receptor, while prolactin binds only to the prolactin receptor (Somers et al. 1994). The C-terminal part of VEGF-C has significant homology to the BR3P protein (Joukov et al. 1996). Also VEGF and VEGF-B_{167} contain two BR3P repeats in their C-terminal domains (see Figure 3), and the NMR structure of the C-terminal domain of VEGF shows that the two repeats indeed fold into two distinct domains (Fairbrother et al. 1998). Nevertheless, apart from the conserved cysteines, the sequence conservation between the C-terminal domains of VEGF and VEGF-C is quite low.

7.3.3 VEGF-C Expression and Its Regulation

As expected for paracrine growth factors, expression of the cognate receptors for VEGF-C and VEGF-D is usually found in close proximity to the growth factor-expressing cells. In the mouse, VEGF-C mRNA expression starts around E8.5 in the head mesenchyme and the developing vertebrae. At E12.5, VEGF-C expression is strong in the mesenchyme of the metanephric and jugular area, where the embryonic lymph sacs sprout from the large veins (Kukk et al. 1996). This pattern is conserved between species. In quail and chick embryos VEGF-C was observed in areas that soon after became rich in lymphatic endothelium (Eichmann et al. 1993; Eichmann et al. 1998). In adult mice, the expression of VEGF-C decreases, but its mRNA can still be found in the lung, heart, liver and kidney (Kukk et al. 1996; Fitz et al. 1997; Lymboussaki et al. 1999).

Although VEGF-D and VEGF-C expression overlaps, e.g. in the heart, important differences exist both during development and adult life, notably the high expression of VEGF-D in the lung (Avantaggiato et al. 1998; Farnebo et al. 1999). The regulation of VEGF-C and VEGF-D expression is less well understood than the one of VEGF (Shweiki et al. 1992; Shima et al. 1996; Stein et al. 1998; Oosthuyse, 2001 #3352). Unlike the VEGF promoter, the VEGF-C promoter lacks hypoxia response elements (Chilov et al. 1997), and thus VEGF-C mRNA levels are not regulated by hypoxia (Enholm et al. 1997). Several growth factors and inflammatory cytokines upregulate VEGF-C expression (Enholm et al. 1997; Ristimaki et al. 1998), whereas steroid hormones act as down-regulators (Laitinen et al. 1997; Ruohola et al. 1999). The VEGF-D promoter has not been characterized extensively, but both hypoxia and cell-cell contacts have been implicated in its regulation (Orlandini and Oliviero 2001; Teng et al. 2002).

7.3.4 VEGF-C Signaling

The signal transduction pathways of VEGF receptors are still not well understood. Endothelial cells typically express more than one VEGF receptor, making it difficult to assign a specific receptor to any biological effect. On the other hand, non-endothelial cells transfected with a single receptor might lack the endothelial cell-specific signal transduction machinery. Only recently has the use of receptor-specific mutants to activate receptors on isolated primary cultures of endothelial cells improved the situation (Gerber et al. 1998b; Joukov et al. 1998; Makinen et al. 2001b).

VEGFR-2

It is thought that in endothelial cells the major VEGF-induced mitogenic signal is routed independently from Ras via PLCγ-PKC to the MAPK cascade (Waltenberger et al. 1994; Seetharam et al. 1995; Cunningham et al. 1997a; Takahashi and Shibuya 1997; Takahashi et al. 1999). Akt phosphorylation by PI3-K seems to be important for survival signaling (Xia et al. 1996; Gerber et al. 1998b; Jiang et al. 2000). This process is dependent on association of VEGFR-2 with vascular endothelial cadherin (Carmeliet et al. 1999a). Akt is also one of the pathways by which VEGF activates the vasorelaxant endothelial nitric oxide synthase (eNOS; Parenti et al. 1998; Dimmeler et al. 1999; Fulton et al. 1999).

VEGF and integrins potentiate each other in a reciprocal manner. VEGF upgregulates the endothelium-specific $\alpha_V\beta_3$ integrin (Senger et al. 1997) and $\alpha_V\beta_3$ integrin associates with VEGFR-2 to potentiate VEGF-induced signaling (Soldi et al. 1999). Other target genes of VEGF that have been implicated in various aspects of angiogenesis include many proteases like plasmin and matrix metalloproteinases (MMPs; Pepper et al. 1991; Unemori et al. 1992), cytoskeletal components such as focal adhesion kinase (FAK; Abedi and Zachary 1997), anti-apoptotic proteins like Bcl-2 (Gerber et al. 1998a) and transcription factors of the STAT family (Korpelainen et al. 1999).

VEGFR-3

Compared to VEGFR-2, little is known about VEGFR-3-initiated signal transduction. From the two splice variants the short isoform is compromised in its signaling capabilities, presumably due to the absent phosphorylation sites of the C-terminus (Pajusola et al. 1993; Fournier et al. 1995). When transfected into PAE cells, VEGFR-3-initiated signal transduction appears very similar to VEGFR-2 (Kroll and Waltenberger 1997), Shc becomes tyrosine phosphorylated, and cell proliferation increases (Pajusola et al. 1994; Fournier et al. 1996; Wang et al. 1997). Like VEGFR-2, VEGFR-3 associates with Grb2 and PLCγ (Pajusola et al. 1994; Fournier et al. 1995; Fournier et al. 1996). Also the biological response is similar in PAE cells: migration, actin reorganization, and proliferation (Joukov et al. 1996; Cao et al. 1998). Only recently have natively VEGFR-3 expressing isolated lymphatic endothelial cells been used to study signal transduction. These studies show distinct differences between VEGFR-2 and VEGFR-3 mediated signaling in the same cell type. E.g. in VEGFR-2 signaling PKC activates p38 MAPK, while in VEGFR-3 signaling p42/p44 MAPK is the target (Gerber et al. 1998b; Taipale et al. 1999; Makinen et al. 2001b).

Outline of the Study

The study presented in this thesis was performed in order to identify ligands for VEGFR-3 and to characterize their structure and function. The hypothesis that VEGF-C is a growth factor for lymphatic endothelial cells was tested using two different in-vivo models. Subsequently, it was shown that a novel VEGFR-3 ligand - VEGF-D - has the same receptor-binding pattern as VEGF-C. Finally, the structural determinants of VEGFR-3 binding were characterized in relation to VEGF. This approach led to the identification of VEGF/VEGF-C mosaic molecules with novel receptor binding profiles, and a panel of these molecules was used to delineate the requirements of specific receptors in the induction of angiogenesis versus lymphangiogenesis in the chorioallantoic membrane.

Materials and Methods

For details see individual articles.

Cell line	Description	Reference	Used in
NIH/3T3-VEGFR-2	mouse embryonic fibroblasts stably transfected with VEGFR-2	(Waltenberger et al. 1994)	III
NIH/3T3-VEGFR-3	mouse embryonic fibroblasts stably transfected with VEGFR-3	(Pajusola et al. 1993)	III
Sf-9	fall army worm *(Spodoptera frigiperda)* ovarian cells	Invitrogen	II, III
High Five	cabbage looper *(Trichoplusia ni)* ovarian cells	Invitrogen	II, III
S2	fruit fly *(Drosophila melanogaster)* cells	Invitrogen	IV
Ba/F3-VEGFR-1-EpoR	mouse pre-B lymphocytes stably transfected with a VEGFR-1-EpoR chimera	(Makinen et al. 2001b)	IV
Ba/F3-VEGFR-2-EpoR	mouse pre-B lymphocytes stably transfected with a VEGFR-2-EpoR chimera	(III; Stacker et al. 1999b)	IV
Ba/F3-VEGFR-3-EpoR	mouse pre-B lymphocytes stably transfected with a VEGFR-3-EpoR chimera	(Achen et al. 2000)	IV
293T	human kidney epithelial cells expressing the SV40 T antigen and the transforming gene of adenovirus 5	American Type Culture Collection CRL-1573	IV
PAE-VEGFR-2	porcine aortic endothelial cells stably transfected with VEGFR-2	(Waltenberger et al. 1994)	IV
PAE-VEGFR-3	porcine aortic endothelial cells stably transfected with VEGFR-3	(Pajusola et al. 1994)	IV

Recombinant protein	Description[1]	Reference	Used in
VEGF-C	mature form	II, III	II, IV
	full length form	III	III
	avian mature form	IV	IV
	mature VEGFR-3-specific mutant	IV	IV
	109 amino acid VHD	IV	IV
VEGF-D	full length and mature forms	III	III
VEGF-A	165 amino acid isoform of VEGF-A	IV	IV
	109 amino acid VHD	IV	IV
VEGF-B	186 amino acid isoform	IV	IV
VEGF-E	viral strains NZ7 and NZ2	IV	IV
HSA	serum albumin	IV	IV
VEGF/VEGF-C mosaics	109 amino acid VHD, produced in both 293T and High Five cells	IV	IV
VEGFR-1_{D1-3}-hIgGF_C	first three extracellular domains of VEGF receptor-1 fused to the F_C domain of human IgG, produced in S2 cells	(Makinen et al. 2001a)	IV
VEGFR-2_{D1-3}-hIgGF_C	first three extracellular domains of VEGF receptor-2 fused to the F_C domain of human IgG, produced in 293T cells	III	IV

VEGFR-3_{D1-3}-hIgGF_C	first three extracellular domains of VEGF receptor-1 fused to the F_C domain of human IgG, produced in 293T cells	(Makinen et al. 2001a)	IV

[1] All proteins are human if not indicated differently.

Antigen	Description	Reference	Used in
vWF	monoclonal antibody against human van Willebrand factor	DAKO	IV
αSMA	monoclonal antibody against alpha smooth muscle actin	Sigma	IV
Prox-1	rabbit antiserum against human Prox-1	(Papoutsi et al. 2000)	IV

DNA vectors	Description	Reference	Used in
pFASTBAC1	baculoviral transfer vector	Invitrogen	III
pFB1-melSP-H_6	baculoviral transfer vector based on pFASTBAC1, modified by addition of the honeybee melittin signal peptide and a hexahistidine tag	II, III, IV	II, III, IV
pSecTagI	dual mammalian/bacterial expression vector based on pSecTagA (Invitrogen); modified by insertion of a bacterial promoter, a tailored MCS and deletion of several restriction sites in the backbone	IV	IV
pKO Scrambler V912	minimal multi-purpose cloning vector	Stratagene	IV
pIgplus, Signal pIgplus	for secreted expression in mammalian cells as a fusion protein with the F_C domain of human IgG	Ingenius	IV
pMT/BiP/V5-His C	for inducible, secreted expression in S2 cells	Invitrogen	IV

Methods	Used in
Generation of transgenic mice	I
Recombinant production and purification of proteins using insect and mammalian cells	II, III, IV
Receptor phosphorylation assay	III, IV
DNA family shuffling	IV
Ba/F3 proliferation assay	IV
CAM assay	IV
Immunohistochemistry	IV
Molecular modeling	IV

Results and Discussion

1 VEGF-C is Lymphangiogenic

VEGF-C was cloned as the ligand for VEGFR-3 (Joukov et al. 1996). Both receptor and ligand expression were suggestive for a role in lymphatic development (Kaipainen et al. 1995; Kukk et al. 1996). On the other hand, VEGF-C induced the proliferation and migration of blood vascular endothelial cells in vitro (Joukov et al. 1996; Lee et al. 1996). To identify its primary in-vivo target we created a transgenic mouse model. Expression of VEGF-C from the human full-length VEGF-cDNA was driven by the keratin-14 promoter (Vassar et al. 1989) specifically in the basal keratinocytes of the skin epidermis.

The choice of the promoter was pragmatic: Expression is local, the skin is easily amenable to observation and manipulation, and the same promoter had been used by Detmar et al. (1998) to create a VEGF-overexpressing mouse.

Transgenic mice with high expression levels could be easily recognized by their smaller size, edematous facial features and disturbed hair-growth. Beneath the epidermis large dilated sinus-like vascular structures devoid of blood cells could be detected. Using immunohistochemistry, electron microscopy and mRNA in-situ hybridization we identified the origin of these structures as lymphatic.

Fluorescence microlymphangiography of the tail showed that the superficial lymphatic network was not altered in its geometry, but that the vessel diameter had enlarged approximately twofold, probably caused by proliferation of the endothelial cells. Remarkably, no sprouting of lymphatics was detected, just like in pathological processes such as lymphangioma or lymphangiectasia. The reason for the absence of sprouting is still unclear. Previous data suggests that the extracellular matrix is instructive in lymphatic vascular proliferation (Clark and Clark 1932) and the extracellular matrix in the superficial lymphatic network might be non-permissive for sprout formation. Apart from VEGFR-3, other receptors were detected on these vascular structures, notably VEGFR-2 and Tie-1.

The high degree of lymphatic specificity was unexpected, particularly as VEGF-C can also activate VEGFR-2. The lack of appropriate proteases might be the cause, since VEGF-C needs to be processed in order to show significant binding to VEGFR-2 (Joukov et al. 1997). Supportive for this explanation is the fact, that in most systems the dominant product of proteolytic processing is the 29/31-kDa form, which binds VEGFR-2 only marginally. Unfortunately, we were not able to demonstrate which form of the protein was predominant in the transgenic mouse skin. The transgene expressed the human protein and although human VEGF-C does bind mouse VEGFR-2, it might do so with a low affinity or it might be less potent due to different receptor internalization kinetics. Lee et al. were unable to immunoprecipitate VEGF-C using mouse VEGFR-2 (1996). There can be considerable interspecies differences of receptor binding affinities and kinetics: human VEGF-D binds VEGFR-2, while the mouse orthologue does not (Baldwin et al. 2001a).

2 Mature VEGF-C is Lymphangiogenic in the Chick CAM

No rapid in-vivo lymphangiogenesis assay has been devised to date. Using the newly discovered VEGF-C, this study characterizes the lymphatics of the CAM and demonstrates its suitability as an in-vivo model for lymphangiogenesis.

To produce the mature form of human VEGF-C, the cDNA coding for the VHD of VEGF-C (nucleotides 658-996, EMBL accession number X94216) was cloned into a baculoviral

transfer vector in between the sequences coding for the honey bee melittin signal peptide (Tessier et al. 1991) and a hexahistidine tag. Protein was isolated from serum-free conditioned supernatant of HighFive cells infected with recombinant baculovirus using Ni^{2+} nitriloacetic acid affinity chromatography.

By evaporation of a sessile drop a radial protein concentration gradient (Deegan 2000) was created on a thermanox cover slip and applied to the differentiated CAM (day 13). Although VEGF-C induced a weak angiogenic response, its main effect was on the lymphatics, which were very abundant in the entire application area. In the region of the highest growth factor concentration a huge lymphatic sinus formed, that could be retrograde-injected with Mercox resin from the lymphatic trunks of the allantoic stalk.

Similar to the results in I, VEGF-C induced proliferation of lymphatic endothelial cells. The existing vessels increased in size, fused with neighboring vessels and formed plexuses. It appeared as if new vessels formed directly under the chorionic epithelium, although the mechanism of formation remained obscure. The mature form of VEGF-C does not require proteolytic processing to allow VEGFR-2 binding. Considering that this form was used, most surprising was again the near absence of any angiogenic effect. Human VEGF-C does bind avian VEGFR-2 (Eichmann et al. 1998) and subsequently the same results have been obtained with avian VEGF-C (own unpublished data). Cao et al. have shown that VEGF-C is angiogenic in the CAM when applied between days 6 to 10.5 (1998). Formation of blood islands commences at E3 in the allantoic bud (Papoutsi et al. 2001), and angiogenesis is considerable until the CAM becomes fully differentiated at day 12. Endothelial labeling indexes peak between days 8 and 10 (Ausprunk et al. 1974). VEGFR-3 is still expressed by the allantoic blood vessels at embryonic day 6 (Eichmann et al. 1993), which equals approximately mouse day 14 (Butler and Juurlink 1987). It is intriguing that avian blood vessels continue expressing VEGFR-3, while mouse blood vessels of a comparable developmental stage have already downregulated it (Kaipainen et al. 1995; Kukk et al. 1996).

It is noteworthy in this context that so far all attempts to achieve a lymphatic phenotype in transgenic mice overexpressing the mature form of VEGF-C have failed (Yulong He, personal communication). It is unlikely, that the lack of the C-terminal tail interferes with correct folding, since biologically active mature VEGF-C can be produced from a truncated cDNA in a variety of expression systems (II; Joukov et al. 1997). The role of the C-terminus of VEGF-C is thus still enigmatic, apart from its apparent function of masking the VEGFR-2 binding epitopes. It does not stimulate tyrosine phosphorylation of VEGFR-2 and VEGFR-3 (Joukov et al. 1997), but might be responsible for the interaction with other receptors, like neuropilin (Makinen et al. 1999; Karkkainen et al. 2001). Based on its homology to BR3P it was suggested that it might regulate the bioavailability of VEGF-C (Joukov et al. 1996).

PlGF was not angiogenic in the CAM in this study. It is difficult to interpret this finding, since PlGF is apparently angiogenic in the early CAM; at least until embryonic day 8 (Ziche et al. 1997; Maglione et al. 2000). Although the avian VEGFR-1 has been cloned (EMBL accession number AB065372), VEGFR-1 specific avian VEGF homologues have not been reported to date.

3 VEGF-D is a Ligand for VEGFR-2 and VEGFR-3

Shortly after the discovery of VEGF-C, two groups reported the cloning of a close VEGF-C paralogue, which was named VEGF-D or *c-fos*-induced growth factor (*FIGF*). Despite the significant homology of FIGF to VEGF

family members, Orlandini et al. chose fibroblasts instead of endothelial cells to test the mitogenic activity of VEGF-D (1996). The high homology of VEGF-D to VEGF-C suggested that VEGF-D might have a similar receptor binding profile and should be tested for binding to endothelial cell-specific receptors.

The truncated cDNA corresponding to the VHD of VEGF-D (nucleotides 651-998, EMBL accession number AJ000185) was cloned into a baculoviral transfer vector as described in II. Full length VEGF-D was expressed in a similar fashion using the endogenous signal peptide of VEGF-D (nucleotides 411-1472, EMBL accession number AJ000185). Conditioned medium was used for receptor phosphorylation experiments, and purified protein was prepared as described in II and used for bioassays. The truncated forms of VEGF-D used in this study corresponded only approximately to the mature endogenous form of VEGF-D, as both predictions of the N-terminal propeptide cleavage site later appeared to be wrong (Stacker et al. 1999a).

Both the truncated and the full-length form of VEGF-D bound to and stimulated tyrosine-phosphorylation of VEGFR-3. However, only the truncated form was able to activate VEGFR-2. The amounts of protein expressed from the full-length cDNA might have been too low to induce detectable receptor phosphorylation. The 29/31-kDa form of human VEGF-D does bind to VEGFR-2 (Stacker et al. 1999a) and baculoviral expression of VEGF-D in insect cells from full-length cDNA results - similarly to VEGF-C - in the uncleaved and the 29/31-kDa forms. Small amounts of the mature 21-kDa form can only be detected after prolonged periods (>72 h) of infection (Hu et al. 1997; own unpublished data). In vivo, both the mature and the 29/31-kDa from were detected from embryonic lung tissue (Stacker et al. 1999a), but VEGF-D might have undergone additional proteolytic processing during lysis and sample preparation.

Surprisingly, it was shown that the receptor binding profile of VEGF-D is not conserved between humans and mice: human VEGF-D binds both VEGFR-2 and VEGFR-3 while mouse VEGF-D fails to bind mouse VEGFR-2 (Baldwin et al. 2001a). This and the fact that mice do not possess the short splice isoform of VEGFR-3 (Galland et al. 1993; Pajusola et al. 1993), but do posses an additional splice-isoform of VEGF-D (Baldwin et al. 2001b), points to potential inter-species differences in lymphangiogenic signaling.

At least in humans, the biochemical properties of VEGF-C and VEGF-D appear interchangeable. Their long forms heterodimerize (own unpublished data). Their expression pattern is overlapping, but not identical, and the keratin-14 transgenic mice show a virtually identical phenotype (Veikkola et al. 2001). Both of them are clearly involved in lymphangiogenesis, but it is not well understood how exactly they divide the labor.

4 Uncoupling of Receptor Binding from Specificity Allows to Create a Super-VEGF

The aim of this study was to perform a screen for structural elements involved in VEGFR-3 interaction, and to comprehensively dissect the effects mediated by individual VEGF receptors. Non-random DNA family shuffling was invented to create a library of mosaic molecules from the two prototype members of the VEGF family – VEGF and VEGF-C. All 512 mosaic molecules of the library were screened for their receptor binding profiles and the results were correlated with their composition to identify structural elements responsible for receptor specificity. The results indicate that all VEGFs bind their receptors in a very similar fashion. Within the VEGF family of

growth factors, specificity is achieved by a limited subset of structural elements of the receptor-binding interface. The bottom groove of VEGF contains critical amino acids that prevent its interaction with VEGFR-3. The corresponding specificity-determining element in VEGF-C is the N-terminal fragment, which prevents interaction with VEGFR-1. These elements are unlikely to contribute much to the binding energy, since by combining the permissive structural elements from both receptors (the N-terminal helix of VEGF and the bottom groove of VEGF-C) mosaic VEGFs able to interact with all three VEGF receptors could be obtained. This concept is schematically explained in Figure 4D. A panel of 10 VEGF mosaic molecules was selected for detailed analysis, including receptor phosphorylation, cell survival using VEGF/Epo receptor chimeras, and in-vivo (lymph)angiogenesis on the chick CAM. To exclude influences of the heparin binding domain of VEGF or the C-terminal domain of VEGF-C, all of the mosaic molecules were applied as minimal peptides of 109 amino acids. VEGFR-2 activation proved sufficient to induce angiogenesis and VEGFR-3 activation sufficient to induce lymphangiogenesis. VEGFR-1 specific mosaics and VEGF-B did not induce any obvious biological effects. The mosaic molecules that showed binding to both VEGFR-1 and VEGFR-3 induced lymphangiogenesis. Their potency though was lower when the affinity towards VEGFR-1 increased, indicating that VEGFR-1 acts also for these artificial molecules as a decoy receptor. None of the mosaic molecules was as potent as the control proteins in the CAM assay and the survival assay using VEGFR-2/EpoR and VEGFR-3/EpoR chimeras. To reach comparable effects in the CAM approximately 5-10 times more protein was applied, even for the most potent mosaics. In the cell survival assay the difference was even more pronounced with the exception of VEGFR-1-mediated cell survival, where several mosaic molecules showed the same potency as VEGF at similar concentrations. Some, but not all of the reduced angiogenic potency can be explained by the lack of the heparin binding domain: also $VEGF_{121}$ is less potent compared to $VEGF_{165}$ (Keyt et al. 1996a).

Usually several rounds of shuffling are performed to create molecules that surpass their parents in one feature (Chang et al. 1999). Although the results using one round of shuffling proved useful to identify structural elements of receptor interaction and specificity, additional optimization is clearly required to convert these molecules into useful research reagents.

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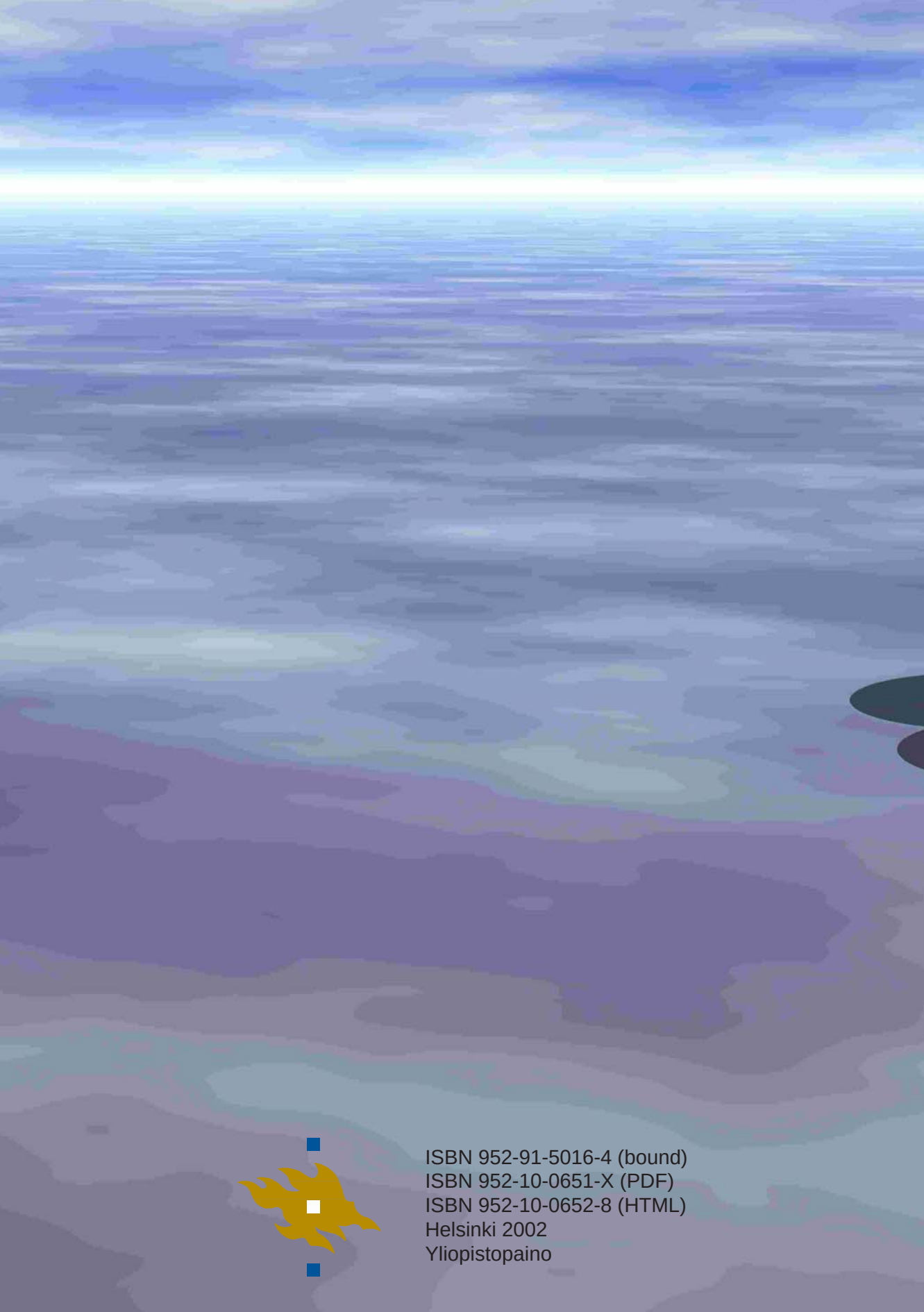

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