Characterization of endothelial cells of lymphatic vessels

Thesis

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Characterization of Endothelial Cells of Lymphatic Vessels.

Sabrina Mancardi

A Thesis submitted for the Degree of Ph. D.
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Cell Biology

International Center for Genetic Engineering and Biotechnology

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And I say that life is indeed darkness save when there is urge,
And all urge is blind save when there is knowledge,
And all knowledge is vain save when there is work,
And all work is empty save when there is love;
And when you work with love you bind yourself to yourself,
and to one another, and to God.

Gibran Kahlil Gibran

To my husband and my son: the
most important experiments of my life.

Preface:

The work described in this thesis was carried out in the laboratory of Molecular Immunology of the International Centre for Genetic Engineering and Biotechnology (ICGEB) of Trieste between November 1995 and May 1998. I am grateful to ICGEB for the provision of laboratory facilities and space. I would like to thank my supervisors for their excellent scientific guidance and moral support throughout this project: firstly Dr. O. Burrone, under whose supervision the experimental work was carried out, and secondly Prof. F. E. Baralle for support my application to the Open University for the Ph.D. course. I am also indebted to Nelson Dusetti, Giorgio Stanta and Marco Bestagno for their friendly help, ideas and discussion throughout this time of exciting work. I am grateful to all the colleagues of my laboratory that in this long period were extremely patient with me. Finally, I would like to thank Giancarlo Lunazzi and his staff for the technical assistance in animal care. This dissertation has no been submitted, in whole or in part, to any other University. This dissertation is a result of my own work and includes nothing, except where stated, which is the outcome of work done in collaboration.

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Abstract

Endothelial cells form the inner lining of blood and lymphatic vessels. In mice only tumours of the blood vessel endothelium (haemangiomas) have been thus far reported. In the first part of this thesis is described a highly reproducible method for the induction of benign tumours of the lymphatic endothelial cells (lymphangioma) in mice, by intraperitoneal injection of incomplete Freund's adjuvant. Different criteria have been used in order to establish the nature of the induced lesion. Morphological and histophatological studies of the tumour developed in the peritoneal cavity revealed the presence of cells at various levels of vascular development. Expression of the endothelial markers PECAM/CD31, ICAM-1/CD54, ICAM 2/CD102 as well as the vascular endothelial growth factor (VEGF) receptor Flk-1, the endothelial cell specific receptors Tie-1, Tie-2, and the lymphatic endothelial specific Flt-4 was identified. When the lesion was induced in β-galactosidase knock-in Flt-4 $^{+/-}$ mice, the tumour endothelia could be stained blue in a number of tumour cells. Tumour-derived cells were propagated *in vitro* where they spontaneously differentiated, forming vessel-like structures. This evidence leads to the conclusion that this is the first experimental protocol for the induction of a lymphatic endothelium hyperplasia in mice peritoneum.

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The second part of this thesis describes the use of this model system to investigate the profile of chemokine expression in murine lymphangiomas and in lymphangioma-derived lymphatic endothelial primary cultures. Chemokines are a superfamily of small, secreted chemoattractant molecules that plays a key role in the immune cell trafficking. Although production of chemokines by vascular endothelial cells has been extensively documented, there is much less information regarding the lymphatic endothelium. The reported results are the first detailed analysis of chemokine production by lymphatic endothelial cells. Chemokines belonging to all three subfamilies (CXC, CC and C), were found to be

expressed in lymphangioma. Among these molecules is remarkable the identification of C10, a molecule previously identified only in the bone marrow.

The molecular as well as functional assays performed provide an indication of the signals that mediate the recruitment of leukocytes into lymphatic vessels.

Chapter 1

<u>INTRODUCTION</u>

The vascular and the lymphatic systems are dispersed organs evolved to provide specific functions such as oxygenation and nutrition to cells throughout the body and the transport of cells necessary to mediate host defence against infections. The lymphatic system, in particular, is also involved in drainage activity of fluids, macromolecules and cells that have leaked into the interstitium. These parallel systems play a significant role in the life of an organism and homeostasis maintenance and consequently are subjects of interest for many investigations. Despite the important anatomical and functional differences between vascular and lymphatic vessels there is a common, central element in both systems: the endothelial cells.

The present thesis is focused on the study of the characterization of lymphatic endothelial cells deriving from tumoral masses induced by intraperitoneal treatment of mice with Freund's adjuvant. This lesion shows the characteristics of lymphangioma as described in humans and is the first report of this kind of tumor in mouse. For this reason it represents a convenient model to investigate the hypothesis that lymphatic endothelial cells produce chemokines which may account for the trafficking and recruitment of leukocytes into the lymphatics.

1.1 The embryonic origin

Vasculogenesis:

The cardiovascular system is the first functional organ system that develops in the vertebrate embryo. Embryonic growth and differentiation essentially depends on transport of nutrients and waste through the early vasculature, and certain events in morphogenesis are thought to be influenced by hemodynamic forces of the beating heart.

The first step in blood vessels formation is the differentiation of vascular endothelial cells, which later on cover the entire inner surface of all blood vessels. During the process of gastrulation, embryonic epithelium (also called inner cell mass in the murine embryo), invaginates through the primitive streak. During this process mesodermal cells are induced by members of the fibroblast growth factor (FGF) family and migrate widely throughout the extraembryonic membranes and the embryo proper (7, 56).

At this stage, during murine embryogenesis, angioblasts arise from the differentiation of mesodermal cells. Angioblast is defined as a cell type that has the potency of differentiating into a endothelial cell but has not yet acquired all the characteristic markers and has not yet formed a lumen. Angioblasts can be first detected in the embryo as early as stage E7.0, and within the yolk sac extraembryonic mesoderm between E7.5 and E8.5 (54, 245)(Fig.1).
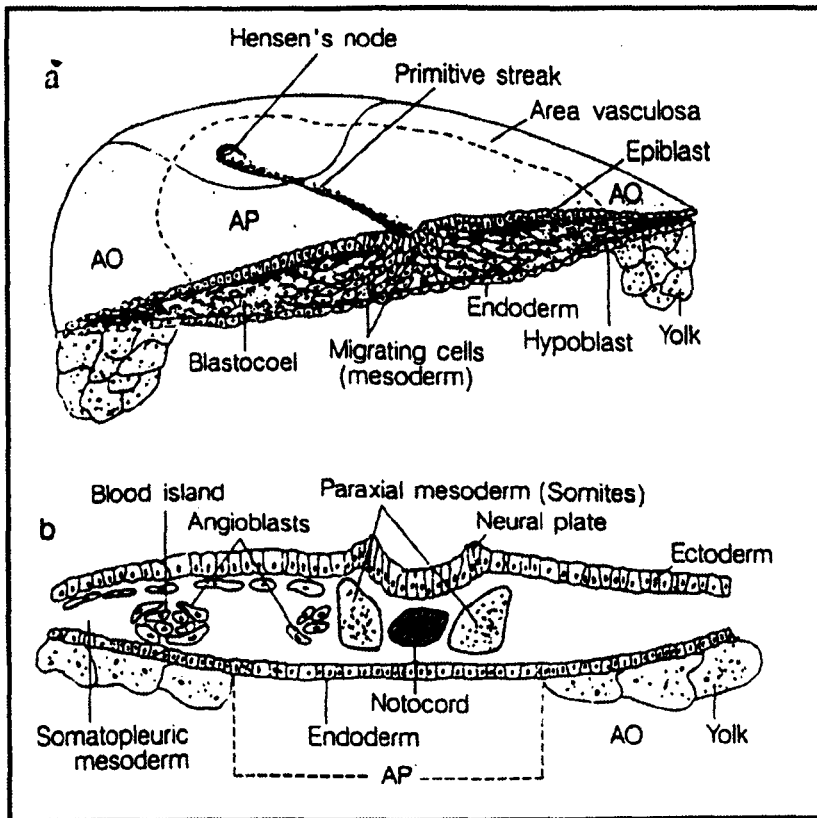
Figure 1. Origin of endothelial cells.

a) Schematic view of mesoderm differentiation and migration in a gastrulating chick embryo after about 17 hrs of incubation. The stippled area indicates the area vasculosa.

b) Schematic cross section of a blood island in the area opaca (AO) and angioblast in the area pellucida (AP) of a 24hrs old chick embryo.

Angioblast are precursors of endothelial and haemopoietic cells. Forming the blood islands haemopoietc precursors are in the internal part, surrounded from endothelial cells precursors.

Reproduction from: Differentiation of endothelium. W. Risau FASEB J. 9,296-933 (1995)

The first intraembryonic angioblasts are seen at the one-somite stage within paraxial and lateral plate mesoderm in close contact with the endoderm (54). From here, angioblastic strands are formed along the lateral edges of the somites, which represents the primordia of the dorsal aorta.

Very shortly after the intraembryonic counterparts have appeared, blood islands emerge in the yolk sac from the splanchnopleuric mesoderm of the area opaca. The peripheral cells of these aggregates are angioblasts (130), whereas the cells in the center of the blood islands are hemopoietic precursor cells.

The formation of these blood islands from *in situ* differentiating endothelial cells is called vasculogenesis. The subsequent processes of blood islands fusion and formation of lumina by angioblasts leads to a primordial vascular network that extends by sprouting of new capillaries (angiogenesis), resulting in an elongated, highly branched vascular plexus. Already at the 2-somite stage the interconnection between this extraembryonic plexus and the intraembryonic vascular primordia is established.

There is a substantial difference between extraembryonic and intraembryonic vasculogenesis process. In the area opaca (yolk sac), endothelial cells differentiation occurs at the same time and in close association with hemopoietic precursors in the blood islands .

The close association of haematopoietic and endothelial precursors cells has led to the assumption that endothelial cells and haematopoietic cells may have a common precursor called haemangioblast. This hypothesis is supported by the observations that several molecules (e.g. CD34, PECAM-

1/CD31, Angiotensin converting enzyme (ACE), von Willebrand Factors (vWF), and P-selectin/ CD62P), are expressed by both cell lineages.

However, within the embryo (zona pellucida), endothelial cells differentiate from the mesoderm as solitary angioblasts without the concomitant differentiation of haemopoietic cells, except for a small region of the aorta (paraaortic clusters)(55). In a second phase, angioblasts migrate and fuse with other angioblasts and capillaries or form vessels *in situ*. This angioblasts differentiation from mesodermal precursors (vasculogenesis) is identified also within organs of endodermal origin (lung, pancreas, spleen), whereas organs of ectodermal or mesenchymal origin (brain, kidney) seem to be vascularized by sprouting of new capillaries from the pre-existing network (226, 252). At this stage, these primitive blood vessels are populated by haemopoietic cells derived from the yolk sac or the paraaortic cluster, while the definitive haematopoietic organs are colonized only by haemopoietic stem cell derived from the paraaortic region.

The process of blood islands induction and subsequent blood vessels formation, therefore can be summarized with the following steps (308, 188):
- contact of either migrating, gastrulating or polyingressing cells bearing growth factor-receptors with growth factor producing cells;
- activation and signal transduction of the growth factor-receptors leading to the activation of genes;
- aggregation of mesodermal cells adjacent to the endoderm (splanchnopleuric or visceral endoderm);

- differentiation of angioblasts (area pellucida) or angioblasts and haemopoietic cells (area opaca and paraaortic clusters) either as a common precursor (haemangioblast) or as two lineages;
- differentiation of endothelial cells, lumen formation, and basal lamina production;
- further growth and migration of endothelial cells and connection of blood islands leading to a primary capillary plexus.

Angiogenesis:

After the primary vascular plexus is formed, more endothelial cells are generated, which can form new capillaries by sprouting or by splitting from their vessel of origin in a process termed angiogenesis. Angiogenesis is the predominant mechanism of blood vessels formation in later stages of embryonic development and in the adult (94), and plays an important role in the remodelling of the primary capillary network into a mature vascular system consisting of vessels of different diameter and functions.

There are at least two mechanisms of angiogenesis :
- true sprouting of capillaries from pre-existing vessels,
- non-sprouting angiogenesis or intussusception.

Sprouting angiogenesis occurs both in the yolk sac and in the embryo (most frequently during later organogenesis, particularly in the brain). There is a proteolytic degradation of the extracellular matrix that is followed by chemotactic migration and proliferation of endothelial cells (232, 244).

Sprouts bearing long filopodia at their tips are then formed which extend from the endothelial lining. Two opposite pre-existing capillaries, meet each other and form a solid strand splitting the intervascular space.

In the process of intussusception, a solid mesenchymal pillar grows into a capillary, subsequently enlarges and forms a new intervascular space. Non-sprouting angiogenesis predominates in organs like lungs that contain intrinsic endothelial precursors and is initially vascularized by vasculogenesis (89, 263) (Fig. 2).

During all the embryological life the vascular system is rapidly remodelled not only to provide the nourishment of the fast-growing embryo, but also to mature in response to specific demands of the developing organs and tissues. Remodelling is initiated by circulating factors and other signals such as shear stress and hyperoxia that are transduced by endothelial cells (240). Modification of the extracellular matrix, secretion of factors with stimulating or inhibiting effects, modification of the expression pattern of specific receptors or adhesion molecules, differentiation of neighbouring cells, lead to the differentiation of angioblast to the quiescent, organ specific and established endothelium of the adult.

In the past decade there has been a significant advance in the understanding of molecular mechanisms regulating vasculogenesis and angiogenesis in the embryo and new blood vessels formation during physiological and pathological processes in the adult.

For these processes growth factors and their receptors play an important role.

Before to approach the molecular mechanisms that underlie these processes, it will be important to summarize as follow:

> The term **"vasculogenesis"** refers to the earliest stage of vascular development, in which vascular endothelial cell precursors undergo differentiation, expansion, and coalescence to form a network of primitive tubules: the primary capillary plexus.
>
> **Angiogenesis** is the process by which, in later stages of embryo development, the primary plexus is remodelled and involves sprouting, branching and differential growth of blood vessels to form the more mature appearing vascular patterns seen in the adult organism. In the adult, all the vascular modifications occur by angiogenesis.

1.2 Molecular mechanisms of Vasculogenesis and Angiogenesis

The molecular mechanisms that underlie vasculogenesis and angiogenesis are still not completely understood, but recently some progress has been made, mainly due to the identification of molecules that are specifically expressed in endothelial cells as soon as they differentiate from the mesoderm. The expression of some of these molecules was investigated in order to define the endothelial origin of the lesion induced in our experimental model.

Fibroblast growth factors

Many evidence (89, 109, 151), support the idea that generation of angioblasts from mesoderm is due to the presence of members of the fibroblast growth factor (FGF) family.

Fibroblast growth factors are a family of nine structurally related polypeptides that are mitogenic for a broad range of cell types as well as mediators of a wide spectrum of developmental and pathophysiological processes in vivo and in vitro (39, 40).

The binding of basic-FGF to fibroblast growth factor receptor-1 (FGFR1) that is expressed on the mesodermal cells surfaces, lead to Ras and MAP kinase activation which phosphorylates transcription factors (TF) resulting in activation of genes necessary for the FGF response (98). After induction by FGF and the consequent proliferation induction of target cells,

Mesodermal precursor

Mesoderm induction

'Haemangioblast'
VEGF-R2[+]

Haematopoiesis

Vasculogenesis

Angioblast
Scl/tal-1[−]
VEGF-R2[+]

Haematopoietic
stem cell
Scl/tal-1+
VEGF-R2+

Vasculogenesis

Primary capillary
plexus

Non-sprouting
angiogenesis
(intussusception)

Sprouting
angiogenesis
VEGF-R2
VEGF-R1
TIE-1
TIE-2
RasGAP

Pruning
Remodelling

VEGF-R2
VEGF-R1
TIE-1
TIE-2
TGFβ-R

SMC
PCT

'Juvenile'
vascular system

Maturation
Remodelling
PDGFβ-R
TGFβ-R

SMC
PCT

Mature
vascular system

Figure 2. Vasculogenesis and Angiogenesis.
Schematic representation of vascular development. The processes (red labels), molecules (green labels) and appearances (black labels) involved in vascular development. Red tips in the primary capillary plexus represent sprouts, yellow circles represent splitting pillars. The haemangioblast as a bipotential precursor is still hypothetical and intermediate steps between some processes have been omitted. Remodelling and maturation is dependent on the tissue and organ context. It is schematized here from the observation in the avian yolk-sac vascular system. A, arteriole, V, venule, SMC, smooh muscle cells, PCT, pericytes. None of the figure is drawn to scale.

Reproduction from: Mechanism of angiogenesis. W. Risau .Nature, 386, 671-674 (1997)

the first molecule known to be expressed in a population of mesodermal cells giving rise to angioblast is the Vascular Endothelial Growth Factor Receptor-2 (VEGFR2) or Fetal Liver Kinase-1 (Flk-1) (193, 315).

In contrast to the crucial function of FGFs and FGF-receptors in mesoderm induction, these molecules do not play a major role in the subsequent morphogenesis of the vascular system in the embryo. In the adult, endothelial cells of larger vessels express FGF-receptors and respond to FGF in vivo suggesting that FGF is probably relevant for regenerative processes but not for vasculogenesis and angiogenesis (174).

Vascular Endothelial Growth Factors and
related Endothelial-specific Receptor Tyrosine Kinases

Vascular Endothelial Growth Factors constitute a family of secreted glycoproteins that have so far been considered as the only growth factors relatively specific for endothelial cells. Almost 10 years ago it was identified VEGF (or Vascular Permeability Factor), the first member of this family (147, 171), a homodimeric glycosylated protein that exists in four isoforms of 121, 165, 189 and 206 amino acids (227). At present four other proteins, placental growth factor (181), VEGF-B (215),VEGF-C (139) and VEGF-D (1), form the endothelial specific family of related growth factors with structural homology to platelet-derived growth factor (PDGF). In particular the cysteines building up the structural fold of these dimeric proteins (three intramolecular disulfide bridges and two intermolecular bridges cross-linking the polypeptide chains) are conserved for these growth factors.

•VEGF is the most critical regulator of endothelial cell proliferation, migration and permeability during embryonic vasculogenesis and, in the adult, in physiological and pathological angiogenesis (78, 87, 284).

VEGF expression by the endodermal germ layer was identified at E7.5 day of the mouse embryogenesis, and was demonstrated that its paracrine secretion supports the differentiation of the VEGFR-2 expressing mesodermal cells to angioblasts (34). It was also reported that a threshold concentration of VEGF is necessary to sustain VEGFR-2 expression: in cells not receiving sufficient signals, a down regulation of the receptor and a lack of differentiation of endothelial cells initially expressing VEGFR2 were described. Finally, mice heterozygous for the VEGF gene disruption die by embryonic day 10.5 or 11.5, indicating a critical dose-dependent embryonic requirement for this growth factor (44, 86) (Table I).

VEGF acts by stimulating members of a family of largely endothelial-specific receptor tyrosine kinases that includes fms-like tyrosine kinase 1 gene (Flt-1)/vascular endothelial growth factor receptor-1 (VEGFR-1) and fetal liver kinase gene-1 (Flk-1)/ vascular endothelial growth factor receptor-2 (VEGFR-2) (64, 238, 298) More recently a third receptor homologous to Flt-1 and Flk-1, designated Flt-4 (VEGFR-3), was identified. It had been previously described as an orphan receptor because it was shown not to bind VEGF (203, 224), while at present it is known to be the specific receptor for VEGF-C (Vascular endothelial Growth Factor-C, or VEGF related protein). The FLT gene family belongs to the class III RTKs (286), which also includes the α and β chains of platelet-derived growth factor receptors (PDGFR), the product of the FLT3/FLK2 gene and two protooncogenes (c-

fms and *c-kit*). The *c-fms* protooncogene was isolated from the feline sarcoma virus. The Flt-4, Flt-1 and Flk-1 RTKs differ from the others members of class III RTKs by having seven instead of five Ig-like loops in their extracellular domains (Fig. 3).

•As previously mentioned, Flk-1 is the first receptor that appears in angioblasts, already detectable in presumptive mesodermal yolk-sac blood island progenitors as early as E7.0 day. During the embryological differentiation Flk-1 become restricted to the endothelial lineage, while at this stage marks both endothelial and haemopoietic precursors cells (193). Mice lacking Flk-1, indeed, die in utero between 8.5 and 9.5 days p.c., as a result of an early defect in the development of hemopoietic and endothelial cells (261). This data indicate that Flk-1 is essential for yolk-sac blood island formation and vasculogenesis in the mouse embryo (Table.I).

•Also the expression of the other receptor for VEGF, Flt-1, was detected during mouse embryogenesis in populations of embryonic cells from which endothelium is derived (230), but the data obtained from mice lacking Flt-1 indicate a role different from the one described for Flk-1. Mouse embryos homozygous for targeted mutation in the *flt-1* locus, form endothelial cells in both embryonic and extraembryonic regions, but these cells are assembled into abnormal disorganized vascular channels, and the animals die in utero at mid-somite stage. Thus, it seems that the Flt-1 signalling pathway is important in controlling normal cells adhesion between endothelial progenitor cells, and/or regulating the interaction of endothelial cells with the extracellular matrix (95) (Table I).

• During the early stages of mouse embryological development also the mRNA expression of the third endothelial-specific receptor, the product of fms-like tyrosine kinase 4 gene (Flt-4), become detectable (140).

In the past years many investigations were performed in order to improve characterisation of this receptor from the biochemical point of view, but its function became evident only when the ligand was identified .

The analysis of RNA isolated from several human and mouse sources showed that Flt-4 is expressed as transcripts of 4.5 and 5.8 kb generated by alternative polyadenylation and alternative splicing, but only the protein translated from the longest form was identified in tissues (223). The two putative Flt-4 forms differ for the presence of an extended carboxyterminal tail of 65 amino acid residues. This tail is probably significant for the protein function because it contains three additional tyrosyl residues that in other receptor tyrosine kinases have been shown to provide autophosphorylation sites important for substrate binding. The Flt-4 precursor is a protein of 170 kDa which upon complete glycosylation becomes a polypeptide of 190 kDa. This is one of the mature forms of Flt-4, a transmembrane protein with seven immunoglobulin like extracellular domains and a intracellular tyrosine kinase domain (Fig. 3). A second form representing the major component, is also found and is composed of two chains of 120 kDa and 75kDa deriving from proteolytic cleavage of the highly glycosylated 190 kDa precursor, linked together by a disulfide bond.

Finally, the activated Flt-4 tyrosine kinase domain was found to interact with the Src homology 2 domains of the SHC and GRB2 adaptor

proteins in vitro and with SHC in cells suggesting that the endothelial cell Flt-4 receptor has a functional tyrosine kinase domain (224).

Flt-4, as above mentioned, is a tyrosine kinase receptor related to the VEGF receptors Flt-1 and Flk-1/KDR, but the observations that VEGF does not show specific binding to Flt-4 nor induce its autophosporylation (203, 224) and that the expression pattern differs from the one of Flt-1 and Flk-1, suggest distinct functions in the regulation of the growth/differentiation of the endothelial network (140).

At E8.5 of mouse development Flt-4 expression is described in the angioblast of head mesenchyme, in the cardinal vein and extraembryonally in the allantois. At 12.5 day p.c. embryos the Flt-4 mRNA signal is detected in developing venous and presumptive lymphatic endothelia, while arterial endothelia become negative. This finding suggests that Flt-4 is a receptor involved in lymphatic vessels development. This hypothesis is supported by the finding that during later stages of development Flt-4 become absolutely restricted to vascular plexuses devoid of red cells, representing developing lymphatic vessels. Although Flt-4 is restricted largely to lymphatic endothelium, mice that contain a knockout of the gene for VEGFR-3 display early embryonic mortality due to the defects in the organization of large vessels prior to the emergence of lymphatics (73) (Table I).

• The expression pattern of Flt-4 suggest that VEGF-C, a member of VEGFs family able to bind and phosphorylate *in vitro* Flk-1 and Flt-4 (139, 158), may function in the formation of the venous and lymphatic vascular system during the embryogenesis. Constitutive expression of VEGF-C in

adult tissues suggests that this molecule is also involved in the maintenance of the differentiated functions of the lymphatic endothelium where Flt-4 is expressed (140). Furthermore, while VEGF-C is homologous to other members of the VEGF/platelet derived growth factor (PDGF) family, its C-terminal half contains extra cysteine-rich motifs reminiscent of the BR3P sequence (69) and characteristic of a protein component of silk produced by the larval salivary glands of the midge, *Chironomus tentans*. Lymphatic capillaries do not have well formed basal laminae and an interesting possibility remains that the VEGF-C silk-like BR3P motif is involved in producing a supramolecular structure which could regulate the availability of the growth factors in tissues (139).

The suggested function of Flt-4 and its ligand VEGF-C was confirmed by the observation that transgenic mice carrying human VEGF-C develop a hyperplasia of the superficial lymphatic network due to the specific over expression of the growth factor. VEGF-C is also a ligand for Flk-1, but the functional significance of this potential interaction *in vivo* is unknown, and in the above transgenic mice it does not induce the sprouting of new vessel (137). From these evidences it seems that VEGF-C induces specific lymphatic endothelial differentiation and proliferation. At present it is not reported the description of an animal model knockout for the VEGF-C gene. Its receptor Flt-4 is so far the only lymphatic-restricted marker described.

• Recently (1), by computer-based homology searching, VEGF-D a new member of the VEGF family was identified. This new growth factor is closely related to VEGF-C, and the analyses of VEGF-D receptor specificity

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revealed that also VEGF-D is a ligand for Flt-4 and Flk-1. Although VEGF-D is mitogenic for endothelial cells in vitro, its in vivo function is still unknown. Because the receptor binding specificities of VEGF-D and VEGF-C are similar, it is possible that also VEGF-D could be involved in the regulation of the growth and/or differentiation of lymphatic endothelium.

A wide view of the role of Vascular Endothelium Growth Factors and related receptors could be summarized as follow:

Flk-1 and Flt-4 are widely expressed during the first stages of embryonic development in endothelial cells. Later, in the fetus, Flt-4 becomes confined to lymphatic endothelial cells (LECs), while Flk-1 is expressed in the vascular counterparts. Because the mature forms of both VEGF-C and VEGF-D can activate these receptors, these growth factors could play roles in coordinating the development of vascular and lymphatic endothelia. It is conceivable that VEGF-C and VEGF-D could serve at a particular site in an embryo to support the growth of both vascular and lymphatic endothelia, whereas expression of VEGF, that activates Flk-1 and Flt-1 but not Flt-4, would play a critical role in the angioblasts commitment and attract the growth of vascular endothelium. Thus changes in the levels of the expression of the genes for VEGF family members could serve to modulate the abundance of different types of vessels in tissue.

Angiopoietin and related receptors

Vascular endothelial growth factor receptors are tyrosine kinase receptors that structurally belong to the PDGF receptors subclasses III, while

FGF receptors form the subclass IV. Both of these subclasses are characterized by extracellular units stabilized by intra-chain disulfide bonds (immunoglobulin like folds). TIE-1 and TIE-2 (also called Tunica interna endothelial cell kinase, TEK), because of their extracellular domains that contain three different types of structural motifs (immunoglobulin (Ig)-like loops, cysteine-rich epidermal growth factor (EGF)-like repeats and fibronectin type III (FNIII) repeats), form a different receptor tyrosine kinase receptor subfamily (72, 228) involved in vascular development (75, 155) (Fig. 3).

Indeed, the onset of embryonic expression for these receptors seems to follow that of the VEGF receptors (71), and the recent generation of mice lacking either TIE-1 or TIE-2 has confirmed the critical requirement of these receptors during blood vessel formation (253). Embryos deficient in TIE-1 expression failed to establish structural integrity of vascular endothelial cells, resulting in oedema and subsequently localized haemorrhage. Mice lacking TIE-1, die between E13.5 and P.0. (Table I).

• Moreover, embryos lacking TIE-2 are more severely affected and die between E9.5 and E10. (Table I). Close observation of TIE-2 deficient mice revealed a malformation of vascular network: in the head region at E9.5, vessels were uniformly dilated and there was no clear distinction between larger and smaller vessels as seen in wild-type embryos, in neuroectoderm the presence of capillary sprouts was not detectable and the vessels that form the myocardial circulation failed to form extensive branches. In the extra-embryonic yolk-sac vasodilatation and abnormal vascular network formation was also observed.

The phenotypic differences between these embryos suggest distinct roles of these two receptors. TIE-1 function is related to endothelial differentiation and the establishment of blood vessel integrity, while TIE-2 is probably involved in the angiogenic processes.

Interestingly, in humans, a missense mutation resulting in an arginine-to-tryptophan substitution at position 849 in the kinase domain of the receptor tyrosine kinase TIE-2 was reported in two disparate families with venous malformations (294). This mutation results in increased activity of TIE-2 and the phenotype originated by the activating mutation in TIE-2 is comprised of vein-like lumens lined by a monolayer of endothelial cells, but with thin walls in which smooth muscle layers are markedly reduced, as compared to normal vessels with similar sized lumens. This observation suggested that TIE-2 receptor expression by endothelial cells is coupled, by some as yet unknown mechanism, to chemotaxis and proliferation of mesenchymal cells and differentiation into smooth muscle cells.

• The recent identification of the specific ligand of TIE-2, termed Angiopoietin-1, allows to elucidate the role of this receptor. Angiopoietin-1 is a secreted 70kDa glycoprotein of 498 highly conserved amino acid residues, which, with a $K_D = 3.7$ nM, binds and activates TIE-2 (60). The addition of this ligand to endothelial cell culture does not show either induction of cells proliferation nor capillaries formation as previously described for VEGF and other classic endothelial growth factors. This observation suggest that *in vivo* the Angiopoietin-1 binding to TIE-2 plays a critical role during angiogenesis regulating the interaction of endothelial cells with the surrounding

mesenchymal cells and the extracellular matrix. This suggestion was confirmed by the observation that loss of Angiopoietin-1 expression in deficient mice leads, in a less severe way, to the same defects described in mice lacking TIE-2 (277) (Table I). Ultrastructural analyses demonstrated the failure to recruit smooth muscle cells and pericyte precursor and the poor association with the underlying matrix. These are probably the major cause in the inhibition of the appropriate vessels stabilization and maturation.

• The vasculature malformations detected in mice due to TIE-2 or angiopoietin-1 deficiency are extremely similar to the ones observed in mice lacking tissue factor (TF), Platelet-derived growth factor receptor-β (PDGFR-β) and TGF-β (44, 172, 246). In these animal models it was reported improper interaction between endothelial and pericytes cells and absence of smooth muscle cells differentiation (Table I). All these data lead to the following general interpretation: the Angiopoietin-1/TIE-2 system plays an important role in vasculature development that differs from the one described for VEGFs and related receptors because it becomes evident in later stages of mouse embryogenesis and does not affect endothelial cells proliferation. Angiopoietin-1 produced by mesenchymal cells activates the TIE-2 receptor on endothelial cells, which in turn leads to the production and/or release of a recruiting signal for mesenchymal cells. Once mesenchymal cells arrive and contact endothelium, TGF-β may be activated (10) and induce differentiation of the mesenchymal cells into pericytes and smooth muscle cells, inhibit endothelial cells proliferation and stimulate matrix deposition. Because TGF-β induces matrix production and modulate integrin expression, an absence of endothelial-smooth muscle cell contacts, with the resulting lack of TGF-

β, may also explain the poor vascular integrity and the reduced remodelling observed in the TIE-2 receptor/ angiopoietin-1-null mice.

Eph receptors and related Ephrin ligands

The Eph receptors comprise the largest subfamily of RTKs, including at least distinct 14 members. The Eph receptors are divergent in sequence from other RTKs and are closely related to cytoplasmic tyrosine kinases within their kinase domains (120) (Fig. 3). All the Eph receptors were initially isolated as orphan receptors, lacking known ligands, but over the past five years at least eight Eph receptor ligands (ephrins) have been described. The ephrins, like their receptor counterparts, are also rather unique among RTK ligands.

The ephrin-Eph system functions in cell-to-cell rather than long range communications, because are attached to the plasma membrane (61, 220). Membrane attachment seems to promote clustering or multimerization of the ligands and it is this clustering that seems to be necessary to activate receptors on adjacent cells (61). Ephrins are divided into two subclasses: the five members of the ephrin-A subgroup (ephrinA1-A5) are attached to the outer leaflet of the plasma membrane via a glycosylphosphatidylinositol (GPI)-anchor, whereas ephrinB ligands (ephrins B1-B3) present a transmembrane region followed by a conserved cytoplasmic domain. Ephrin A and ephrinB molecules bind specifically the corresponding EphA and EphB receptor subfamilies respectively whereas, within these subfamilies, interactions are highly promiscuous (32, 100).
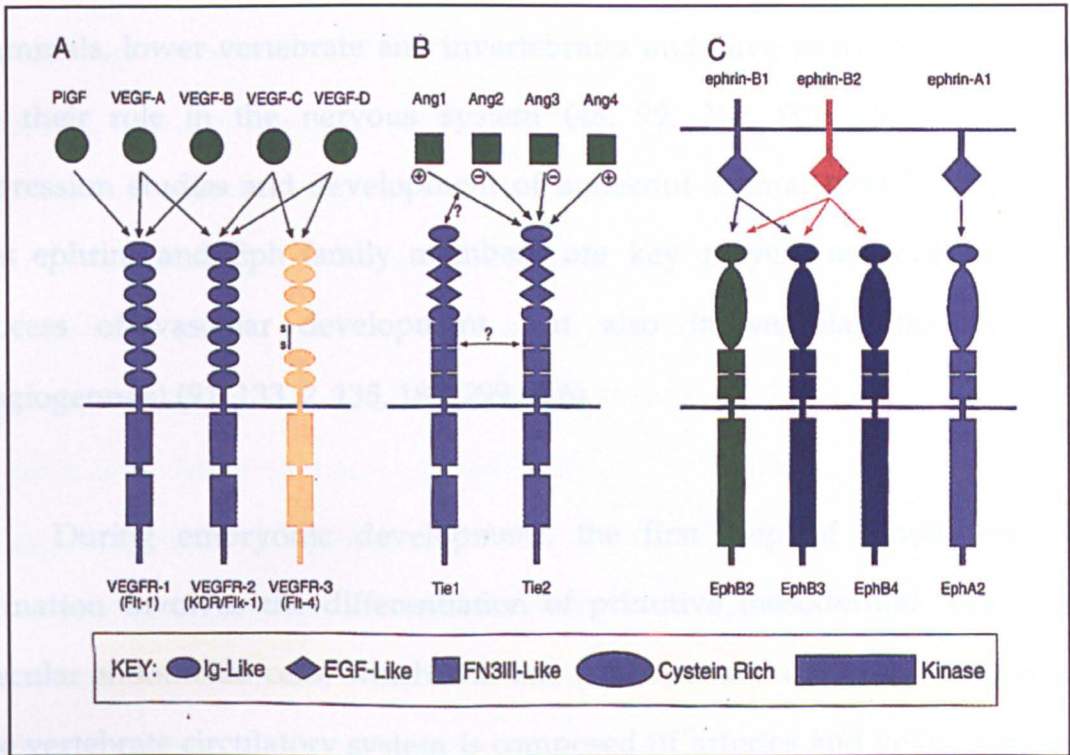
Figure 3. Ligands and RTK families involved in vascular development.

A)   VEGFS and the VEGF receptors.

B)   The angiopoietins (Ang) and the Tie receptor family.

C)   The ephrins and the Eph receptors.

Arrow indicate documented interactions among ligands with their receptors. Receptors and ligand structures are drawn colour coded to indicate their domains of expression; purple represents proteins found expressed on both arteries and veins. Red and blue structures are found on arteries and veins, respectively; yellow denotes expression on lymphatic vessel and green structures represents receptors or ligands expressed in tissue surrounding blood vessels.

These receptors and their cell-surface-bound ligands, play an important roles in a variety of processes during embryonic development of mammals, lower vertebrate and invertebrates and have been most studied for their role in the nervous system (48, 99, 219, 90). Most recently, expression studies and development of knockout animal models establish that ephrins and Eph family members are key players not only in the process of vascular development, but also in vascular remodelling (angiogenesis) (91, 133, 2, 135, 189, 299, 316).

During embryonic development, the first step of blood vessels formation involves the differentiation of primitive mesodermal cells into vascular endothelial cells, which will line the internal surface of all vessels. The vertebrate circulatory system is composed of arteries and veins: arterial and venous endothelial cells are molecularly distinct from the earliest stages of angiogenesis. This distinction is revealed by expression on arterial cells of ephrin-B2 (21, 22), whose cognate receptor Eph-B4 (8) is expressed on venous cells as demonstrated following the expression of *ephrin-B2-LacZ* gene (299). Ephrin-B2 knockout mice display defects in angiogenesis by both arteries and veins in the capillary networks of the head and yolk sac as well as in myocardial trabeculation: the deficient mouse dies in E10.5 (Table I). These data are suggesting that differences between arteries and veins are in part genetically determined and reciprocal interactions between pre-specified arterial and venous endothelial cells are crucial for the morphogenesis of the capillary bed and angiogenesis mechanism (299, 316). In mice lacking ephrin-B2 was also observed that yolk mesenchymal and peri-endothelial cells appeared to be poorly associated with the endothelium. Defects in later

vascular remodelling as well as in heart trabeculation, due to the altered interactions of endothelial cells with support cells, was highly reminiscent of defects observed in mice lacking Ang1 or its receptor Tie2 (253, 277).

Recently was also found that other B-class receptors and ligands displayed expression patterns overlapping those of ephrin-2 and EphB4 and play critical roles in vascular development (2). Embryos lacking both EphB2 and Eph3 (EphB2/EphB3) with a penetrance of 30%, exhibited defects in vascular development that largely phenocopy those of the ephrin-B2 knockouts: defects in the primary plexi of the yolk sac and head and defective heart morphogenesis but with a normal sprouting angiogenesis. Ephrin-B1 is co-expressed with ephrin-B2, on arterial endothelium, though its presence there is not sufficient to compensate for the knockout of ephrin-B2 (2), and also expressed in veins. At present the EphB4 knockout model is not officially described, but a personal communication of M. Aguet to N.W. Gale (101), reported that mice lacking EphB4 display embryonic lethal phenotype, probably due to vascular defects in angiogenesis. At present also the ephrin-B1 knockout mice is not available.

Gene knockout	Time of Death	Stage of vessel Development	Causes of mortality	References
VEGF-A(+/-)	E11.5	Vasculogenesis/ (Angiogenesis)	Reduced red blood cells; defective heart and aorta formation; defective vessel connectivity; defective sprouting.	Carmaliet et al. (1996); Ferrara et al. (1996).
VEGF-A(-/-)	E10.5	Vasculogenesis	Absent dorsal aorta; defective endothelial cell development.	Carmaliet et al. (1996); Ferrara et al. (1996).

VEGFR-1	E8.5-E9.5	Vasculogenesis	Failure of endothelial cell formation.	Fong et al. (1995).
VEGFR-2	E8.5-E9.5	Vasculogenesis	Excess endothelial cells form abnormal vessels structures entering vessel lumens.	Shalaby et al. (1995, 1997).
VEGFR-3	E10.5-E12	Vasculogenesis	Defective vessel remodelling and organization; irregular large vessels with defective lumens.	Dumont et al. (1998)
Ang1	E10.5	Angiogenesis	Defective vessels remodelling, organization, and sprouting; heart trabeculation defects.	Davis et al. (1996); Suri et al. (1995).
Ang2	E12.5-P1	Maturity	Poor vessel integrity, edema, and haemorrhage.	Maisonpierre et al. (1997).
Tie1	E13.5-P1	Maturity	Poor vessel integrity, edema, and haemorrhage.	Puri et al. (1995); Sato et al.(1995).
Tie2	E10.5	Angiogenesis	Defective vessel remodelling, organization, and sprouting; heart trabeculation defects.	Dumont et al. (1994); Sato et al. (1995).
Ephrin-B2	E10.5	(Vasculogenesis)/ Angiogenesis	Some defective vessel primordia; defective vessel remodelling, organization, and sprouting; heart trabeculation defects.	Wang et al. (1998); Adams et al. (1999).
EphB2/EphB3	E10.5 (approx.30%)	(Vasculogenesis)/ Angiogenesis	Some defective vessel primordia; defective vessel remodelling, organization, and sprouting; heart trabeculation defects.	Orioli et al. (1996); Adams et al. (1996).
EphB4	E10.5	?	?	Aguet (in prep.)

Table I: Knockout animals from the VEGFR/VEGF, Tie2/Ang, and Eph/ephrin families exhibited a variety of embryonic defects in vascular development

1.3   Role of Cell Adhesion Molecules

During embryonic development endothelial cells differentiate from a common precursors called angioblast and acquire organ-specific properties. One of the important determinants of endothelial differentiation is the local microenvironment, and especially the interaction with surrounding cells. This interaction may occur through the release of soluble cytokine, cell-to-cell adhesion and communication, and the synthesis of matrix protein on which the endothelium adheres and grows. To date four families of cell adhesion molecules have been described: integrins, immunoglobulin superfamily members, cadherins and selectin. Members of each family have been detected in angiogenic vessels.

Some molecules are expressed in very early stage of development, other appear only in advanced stages and are related to a mature, functional differentiation level of the cells, and other are required during the differentiation process in order to drive the correct endothelial maturation.

•PECAM-1, Vascular endothelial cadherin (VE-cadherin/cadherin 5), and CD34 are cell-cell adhesion molecules expressed very early in angioblasts. Although PECAM-1 and CD34 are also present in different haemopoietic cell types, VE-cadherin is most specific for endothelium (15, 162, 322). The function of the mucin-like CD34 molecule in embryonic capillaries is not well understood, while VE-cadherin and PECAM-1 are probably involved in homotypic endothelial cell adhesion and in the

formation of inter-endothelial junctions, which are important for lumen formation, cell polarity and vascular permeability.

• During vasculogenesis fibronectin and its receptor $\alpha5\beta1$ integrin are required for vascular development and mouse embryo lacking their expression die in utero for early vascular defects (104, 317). In addition, the $\beta1$ integrin is important in vasculogenesis and lumen formation of the dorsal aorta(70). More recently the integrin $\alpha V\beta3$ was found to be upregulated in angiogenic endothelium and seems to play a determinant role in tumour angiogenesis (36).

• Laminin, vitronectin and other extracellular matrix molecules are produced by endothelial cells in later vasculogenesis stages, and an intact basal lamina is a characteristic feature of a mature blood vessel (245). These molecules may not only have important functions in cell adhesion but also in the storage, accumulation and activation of proteases and proteases inhibitors such as urokinase and plasminogen activator inhibitor-1, and growth factors such as VEGF and cytokines (280).

• Intercellular adhesion molecule-1 and -2 (ICAM-1/CD54 and ICAM-2/CD102) are members of the immunoglobulin gene superfamily identified as counter receptors of lymphocyte function-associated Ag-1 (LFA-1). LFA-1 is a member of the integrin family that has an α/β heterodimer structure and it is expressed on almost all types of leukocytes (268, 271) Important adhesive interactions during inflammatory and immune responses are mediated by LFA-1 and its ligands.

ICAM-1 is a single chain glycoprotein with a polypeptide core of 55kDa and it is characterized by five extracellular Ig-like domains. ICAM-1 can be expressed on many haematopoietic and non-haematopoietic lineages such as thymic epithelial cells, fibroblast, vascular endothelial cells, lymph nodes and Peyer's patches. In endothelial cells the constitutive ICAM-1 expression is rather low, but can be induced or upregulated by inflammatory mediators such as IL-1, tumour necrosis factor (TNF) and interferon-γ (IFN-1) (187, 234). Identification of ICAM-1 expression in unstimulated yolk sacs and in cystic embryoid bodies (CEBs) and the increasing of the signal after exposure to LPS (127), implies an early ontogeny for the endothelial cell signal transduction pathway necessary for leukocyte recruitment and suggests a developmental role for this adhesion receptor.

ICAM-2 is a 60kDa surface glycoprotein member of the Ig-superfamily with two immunoglobulin-like extracellular domains. Also ICAM-2 is broadly distributed on haemopoietic lineage, in particular on resting lymphocytes and monocytes, but, in comparison with ICAM-1, its expression in tissues is highly restricted to vascular endothelium. Basal expression of ICAM-2 on endothelium is much stronger than on leukocytes and much higher than that of ICAM-1 and is not further increased by inflammatory mediators (62). The interaction of LFA-1 with ICAM-2 seems to be a major component of lymphocytes adhesion to unstimulated cultured endothelium (77). Because ICAM-2 is expressed on both high endothelial venules in lymph nodes and on vascular endothelium in other tissues, it has been hypothesized that ICAM-2 may play an important role in lymphocyte recirculation (62). At present there are no evidences of ICAM-2 participation in vasculogenesis or angiogenesis processes, while some reports indicate a

down-regulation of its expression in tumour associated endothelium (112, 113).

Thus, ICAM-1 and ICAM-2 are two endothelial markers: while ICAM-1 is inducible and its expression levels related to a functional state, and ICAM-2 shows constitutive expression.

1.4 Endothelial Cell:

Heterogeneity and different Culture Models

Endothelial cells, forming the lining of venous and lymphatic vessels, actively participate in many important functions in order to control specifically the functional homeostasis of the different organs. During the differentiation process, endothelial cells acquire specialised properties and morphological characteristics. How endothelial cells take different pathways of differentiation is still unclear, but one of the important determinants in this process, as above mentioned, is the local environment and the interaction with surrounding cells.

Thus, a unique characteristic of endothelial cells is that, although they present many common functional and morphological features, they also display remarkable heterogeneity in different organs (19). Even in the same organ, the endothelium of venous and lymphatic, of large and small vessels, veins and arteries exhibits significant differences. Moreover, embryonic and adult endothelium is not equally susceptible to differentiation factors (12, 243). The adult endothelium, nevertheless, can reversibly modify its functions on activation (i.e. after exposure to inflammatory cytokines), and consequently display different and reversible phenotypes (184). Finally,

morphological features and responses of endothelial cells are modified also in senescence, a mechanism that is still poorly understood (102).

The identification of specific markers expressed by endothelial cells is essential for the recognition of these cells and their different phenotypes *in vivo* and *in vitro*. At present several molecules suitable for this purpose are known.

A number of these molecules, that includes Factor VIII-related antigen, Angiotensin converting enzyme, von Willebrand Factor, thrombomodulin, some integrins, PECAM-1, VE-cadherin, are well characterized, but in most cases, when markers were identified by monoclonal antibodies directed to endothelial cells (e.g. MECA-32, EN4, PAL-E), the antigens were not molecularly characterized (20, 81, 119, 162, 291, 305, 257, 258).

Most of these endothelial markers are constitutive and essentially present in all types of endothelium. Other molecules are expressed only after activation by inflammatory cytokines, growth factors or related to the functional stage during vasculogenesis or angiogenesis (ICAM-1, VCAM-1, E- and P-Selectin, Flk-1, Flt-1, Tie-1) (144, 288, 268, 230, 203). Although an evident improvement in this field over the recent years, the number of markers rather specific for endothelial cells of different origin, is relatively scarce. Different markers has been identified for the characterization of highly specialized endothelium such as the high endothelium present in the postcapillary venules (HEVs) of lymphnodes and Peyer's patches (GlyCAM-1, Meca-79), the endothelium of the bone marrow (GM-CSF, PDGF, different interleukines) or the endothelium of the brain (HT7, Glut-1) (163, 182, 191, 260). Nevertheless the number of helpful molecules for the characterization

of microvascular endothelial cells or lymphatic vessels endothelial cells is particularly limited. To date, the only known marker specific for lymphatic endothelial cells is the VEGF-C receptor Flt-4 (140).

The isolation of endothelial cells from the microvasculature of certain vascular regions as well as from lymphatic vessel is still experimentally difficult and this is probably one of the major reasons for the present difficulties in identifying new specific markers. Another limitation to the study of endothelial cell is the fact that, once in culture, these cells tend to lose their specialized properties. For all these reason, over recent years, many efforts have been made to develop new techniques to isolate and culture specific endothelial cells in order to create more realistic *in vitro* models. Even though, isolation and culture of endothelial cells from large vessels (e.g. human umbilical vein (HUVEC), bovine and porcine aorta (BAE, PAE) has become a routine procedure, endothelium cells from other sources are at present being investigated, such as human dermal microvascular endothelial cells (HDMEC), endothelial cells isolated from murine brain, lung or liver, from lymphnodes or Payer's patches are only few examples of the many available (19, 241).

Moreover, to avoid the phenotypical modifications that occur in primary culture new culture strategies are developed: e.g. use of specific extracellular matrix, of selective media or coculture with other cell types in order to reproduce in vitro specific microenvironment (66, 76, 117, 129). Several effort have been also done for the establishment of cell lines of transformed endothelial cells (41, 96, 131). These cell lines represent useful models that allow in vitro studies to investigate the biology and the heterogeneity of

endothelial cells. To improve the growth characteristic of primary cells in culture different constructs containing the simian virus 40 large T oncogene, polyoma middle T antigene or the E6/E7 genes of human papilloma virus have been used.

The integration of this exogenus DNA, beside growth stimulation and immortalization, often induces changes in the karyotypes and in the phenotypic features of the cells. At present, many recent studying aim at obtaining conditions that could eliminate these undesirable effects.

It is also important to mention briefly the recent progresses in the establishment of *in vivo* model in order to investigate neovascularization and angiogenetic processes.

The quantification of neovascularization in vivo has been performed primarily by means of the following three approaches:

- microcirculatory preparations in animals, mostly chick embryo and
    rodents;
  - vascularization into biocompatible polymer matrix implants;
  - excision of vascularized animal or human tissues.

Several advantages and different limits are evident for each of these approaches, and the present research is focused on developing angiogenic assays highly reproducible and quantitative. This aspect become relevant in order to study the angiogenic/antiangiogenic activity of different growth factors or other substances (e.g. chemokines, extracellular matrix, proteases, steroids hormones, some lipids) that seem to play a role in the establishment of "angiogenic diseases" (polyartritis, psoriasis, diabetic retinopathy, endometriosis) or in tumoral angiogenesis (92-94).

As a final remark, it is relevant the development of transgenic/knockout mouse models that has provided powerful insights into molecular mechanisms of angiogenesis.

1.5 Angiogenesis and Vascular Disorders

Angiogenesis, as previously described, is a fundamental process by which new blood vessels are formed from the established microcirculation. It is essential and particularly active in a large number of normal processes during pre- and post-natal life. In the adult organism, in which the turnover of endothelial cells is extremely low and the circulatory tree is established, it appears only in specific circumstances: reproduction, wound repair, inflammation and collaterization in response to ischaemic stimuli. Under these conditions angiogenesis is highly regulated, i.e. turned on for brief periods (days) and then completely inhibited. The process of neovascularization is extremely complex and implicate the following event:

Endothelial cells and pericyte activation;

basal lumina degradation;

migration and proliferation of endothelial cells and pericytes;

formation of a new capillary vessel lumen;

appearance of pericytes around new capillaries;

development of a new basal lamina;

capillary loop formation;

persistence or involution and differentiation of the new vessels;

capillary network formation;

organization into larger microvessels.

It is evident that a number of different factors participate in the control of angiogenesis *in vivo* and the control derives from the balance between stimulatory and inhibitory substances. The angiopoietic activities of FGF, VEGF, PDGF, the E series of prostaglandin, angiogenin and monobutyrin as well as the nicotinamide and related compounds are extensively described (93). In several cases the molecular mechanism is completely understood, in others it is still under investigation. Generally, angiogenic factors act on the proliferation and/or the motility of endothelial cells.

By contrast, it has been demonstrated that angiostatin, endostatin, platelet factor 4, interferon-alfa, interferon-inducible protein 10 and some steroids (9, 118, 183, 212, 274) may inhibit the proliferation of endothelial cells and consequently are described as antiangiogenetic molecules. The identification of these molecules and the recognition of this biological activity lead to an increased interest for the investigation in this field in order to develop new therapeutic strategies.

Thus, many diseases are driven by persistence of unregulated angiogenesis, probably due to the failure of a such complex balance.

In arthritis, new capillary blood vessels invade the joint and destroy cartilage. In diabetes, new capillaries in the retina invade the vitreous, bleed and cause blindness. Other frequent ocular diseases related to angiogenesis are retrolental fibroplasia and neovascular glaucoma. The excessive proliferation and shedding of epidermis evident in psoriasis, may depend on abnormal capillary growth in the dermis, and capillary proliferation within arteriosclerotic plaques which may bleed, contribute to sudden occlusion of coronary arteries (93). It is at present absolutely evident that tumor growth

and metastasis are angiogenesis-dependent (94, 192). A tumour continuously stimulates the growth of new capillary blood vessels for the tumor itself to grow (143). Furthermore, the new blood vessels embedded in a tumour provide a gateway for tumor cells to enter into the circulation and to metastatize to distant sites, such as liver, lung or bone. Endothelial cells can also originate tumours for itself with a more or less severe clinical outcome.

Hemangioma, in which abnormal capillary proliferation appears in newborn babies and may persist for up to two years, can in some cases cause death from haemorrhage or spontaneously regress. Angiofibroma is a more severe tumour and appear in the nasopharynx usually in adolescents.

Kaposi sarcoma (KS) is probably one of the most severe angioproliferative disease and recently, for its association to AIDS, also relatively frequent. KS lesions display intense endothelial cell proliferation, disorganized angiogenesis with migratory spindle cells, interstitial sclerosis, often progressing to an angiosarcoma-like stage resembling the Stewart-Treves syndrome associated with longstanding lymphedema (309). AIDS-KS patients commonly die with massive pulmonary edema, serus effusions and anasarca.

Human herpesvirus-8 (HHV-8) has been detected in Kaposi's sarcoma lesions of all types (AIDS-related, classical and endemic), and consequently has been referred to as Kaposi's sarcoma-associated herpesvirus (KSHV) (196).

Cumulative data from epidemiological and serological studies indicate that infection by human herpesvirus-8 (HHV-8) precedes development of KS (262, 307) and recently was suggested that the expression of KSHV vBcl-2 may play an anti-apoptotic role in virus infected cells (251, 290) promoting

the establishment of the disease. Although these relevant progresses, the cell of origin and the nature of KS (multicentric vascular hyperplasia vs. neoplasm) remain mired in controversy. The "KS cell" is considered to arise from blood vascular endothelial variants or (most often mentioned) from lymphatic (17).

Whereas the recent explosion of interest in angiogenesis has focused almost entirely on the regulation of blood vessel growth and what turns this process on and off, it is becoming evident that similar processes and probably similar underlying physiochemical signals and mediators must be involved also in normal and pathologic lymphangiogenesis (Fig. 4).

1.6 Lymphangioma

Disordered lymphangiogenesis range from strangulating cystic hygroma, vascular birthmark (Klippel Trenaunay-Weber) syndrome, Kaposi sarcoma and Stewart-Treves (lymph)angiosarcoma. Lymphangiomas, like their blood vessels counterparts (haemangioma), are common disfiguring tumors of childhood and may also arise and enlarge rapidly in adulthood (170, 200). Some lymphangioma syndromes follow mendelian inheritance (autosomal dominant), whereas others are dysmorphogenic conditions that may represent somatic mutations. In vivo behaviour and aggressiveness varies between a ductal malformation (e.g. cystic hygroma) and true neoplasia ("aggressive" lymphangioma) as these tumors insinuate into adjacent tissues and encroach on vital organs. Some lymphangiomas may be multifocal or multicenter and occasionally malignant transformation takes place (e.g. Maffucci syndrome). Sex hormones, such as exogenous or
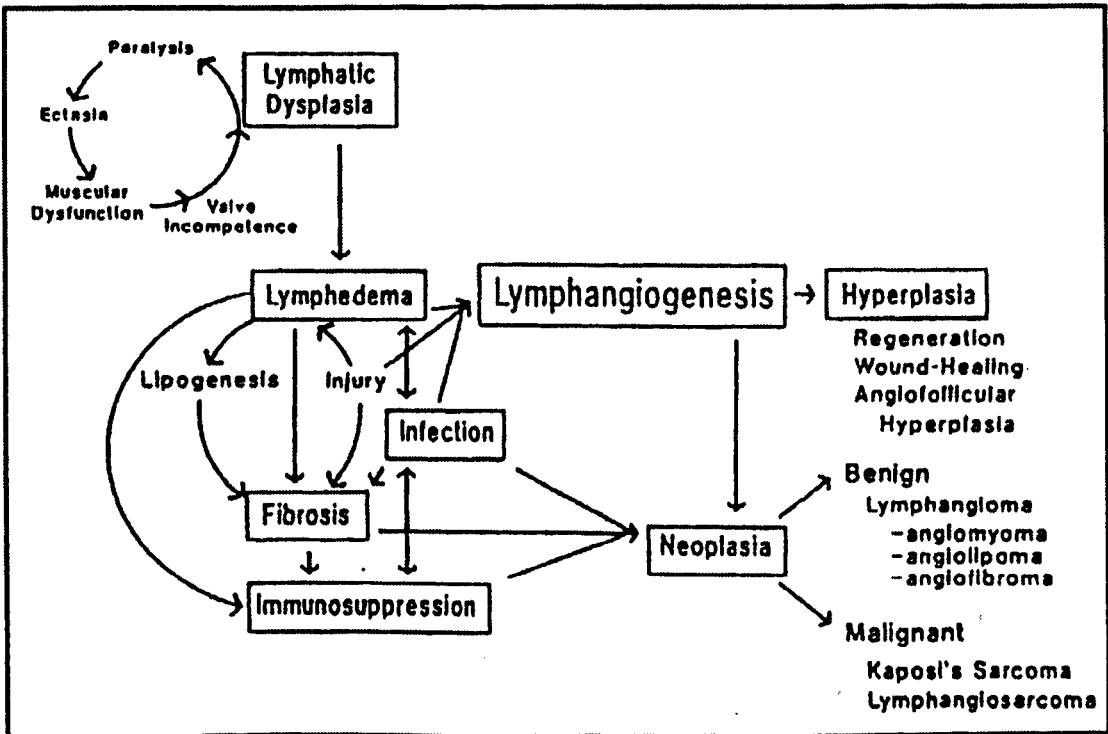
Figure 4. Lymphangiogenesis disorders.

Hypothesized links between the pathophysiologic events and sequelae of lymph stasis and disturbed lymphangiogenesis, hyperplasia, displasia and neoplasia. During a latent period (no overt edema), lymphatic truncal dysplasia is accompanied by lymphatic ectasia, muscular dysfunction, lymphangiogenesis and intraluminal valve incompetence culminating in a persistent protein-rich lymphedema (more than 1.5g/dl). Superimposed opportunistic infection, immunosuppression, and injury with repeated lymphangitis promote intractable lymphedema, intense (lymph)angiogenesis, lipid deposition and fibrosis, and on rare occasions malignant vascular neoplasia. This sequence of events may evolve as a local phenomenon as in peripheral lymphedema ("local AIDS") or as a multitude of isolated or generalized visceral disorders characterized by scar formation and angiohyperplasia and neoplasia including AIDS-related syndromes.

endogenous estrogen (oral contraceptives and puberty hormones) appear to stimulate lymphangioma and other angiotumor growth.

The *in vivo* behaviour of these tumors, characterized by formation of tubes, cystis and other dysplastic structures, can be reproduced *in vitro* (303, 304). Whereas normal endothelium from vein, artery, lymphatic and microvasculature in vitro exhibits typical contact-inhibited homogeneous cobblestone morphology, dysplastic and neoplastic endothelium exhibits a more heterogeneous cell population of multiple phenotypes and absence or reduced contact-inhibition. This is probably due to the presence of transformed phenotype. Lymphangioma derived cells, propagated for long periods in culture, exhibit spontaneous lymphangiogenesis in vitro, retaining the morphologic characteristic of lymphatic endothelium, albeit with great heterogeneity. The percentage of the cell population exhibiting vWF positivity is also extremely variable (168).

These *in vitro* models are not only useful to study endothelial cell structure and function, but also to define the biological behaviour of transdifferentiated or transformed lymphatic endothelium.

At present, this tumor has been described only in humans, while in mice only haemangiomas have been reported (124).

1.7 Freund's Emulsified Mineral Oil Adjuvants

Historically, the development of Freund's adjuvant emerged from studies of tubercolosis in which it appeared that the immunologic responses in animals to various antigens were enhanced by introduction into the animal living *Mycobacterium tubercolosis*. Moreover, in the presence of

Mycobacterium, the reaction of hypersensitivity obtained was of the delayed type, transferable with leukocytes. In studies to measure the effect of mineral oil in bringing about delayed-type hypersensitivity to killed Mycobacteria, Freund noted that there was a remarkable increase in complement-fixing antibodies response, as well as in delayed hypersensitivity reaction. These basic principles were applied to improve immunologic responses to various added antigens and finally the Freund's adjuvant were evolved. Essentially, Freund's adjuvant consist of a water-in-oil emulsions of acqueous antigen in paraffin (mineral) oil of low specific gravity and low viscosity. Drakeol 6VR is a commonly used mineral oil and Aracel A (Mannide monooleate) is employed as emulsifer. There are two Freund's adjuvants: complete and incomplete, differing for the presence (in the complete formulation) of dried, heat-killed *Mycobacterium tubercolosis* or *butyricum*.

The discovery of the adjuvant action of Freund's mineral oil emulsions fulfilled an important need for increasing antibody response to antigens and, at the moment in which it was not yet developed the modern concept of safety, found rapid application to experimental vaccines in man (97, 125). Only later, in order to assess the safety of Freund's adjuvant, several investigation were performed in animals.

Several publications report various harmful effects following administration of Freund's adjuvant or its component (18, 126, 165). Disseminated granuloma were recorded to be formed in the lung, liver and kidney of rabbit treated once weekly for four weeks by intracutaneous route with Aracel A in physiological saline solution and light mineral oil (273). Granulomatous proliferative lesions were described in the liver and spleen of

mice, in the lungs of guinea pigs and in the lung, brain and liver of hamster which received the oil-saline mixture (165). In 1960 and 1961, Potter and Robertson and Lieberman et al. (173, 236) respectively have recorded appearance of ascites and plasma-cell proliferation in mice of different strains following repeated intraperitoneal injection of incomplete Freund's adjuvant emulsified with physiological saline solution or with a suspension of *Staphylococcus aureus*. The injection of the adjuvant preparations as well as of mineral oil alone was able to induce in mice of BALB/c strain plasma-cell neoplasm, with the consequential development of myeloma serum and Bence-Jones protein in the urine.

The tumours in certain mice appeared to evolved from lipogranulomatous tissue, appearing in the form of multiple nodules in the mesenteries and on the peritoneal surfaces (173).

Beside these studies model, Freund's adjuvant was used also to assess experimental model of diseases with a immunological origin. Administration to animals of autologous, isologous, homologous or even related heterologous tissue in Freund's adjuvants gives rise to various experimental illnesses, termed autoallergic disease, which serve as a model for similar and probably related autoimmune disorders in humans (297). The injection of Freund's adjuvant into the foodpads of some rat and mouse strains, has been recognized as a useful animal model to study arthritis (150, 239).
Finally, Freund's adjuvant is widely used in different routes and schedules of immunization in animals for the production of monoclonal antibodies.

The Chemokines

Endothelial cells line the internal surfaces of blood and lymphatic vessels and have long been considered only as a "passive" lining. They were attributed negative properties, i.e. to act as a non-thrombogenic substrate for blood and were considered to participate in tissue reactions essentially as a target for injurious agents. Isolating and culturing endothelial cells from various tissues allowed not only in recognition of the wide heterogeneity of endothelial cells, but also in studying their complex reactions to a variety of activating stimuli. Endothelial cells have now emerged as active participants in many physiological and pathological processes.

Haemostasis, inflammatory reactions, and immunity involve close interactions between endothelial and immunocompetent cells . In particular ontogeny and leukocyte trafficking require an intimate relationship between these two kinds of cells. The bidirectional interactions between leukocytes and endothelial cells is mediated by cytokines (184, 186).

In the large and complex cytokine superfamily an important role is carried out by a class of molecules with very distinct effects and characterized by common structural feature: the chemokines.

1.8 General Features of Chemokines

Chemokines constitute a superfamily of small, inducible, secreted proinflammatory cytokines involved in a variety of immune responses and

primarily described as chemoattractans and activators of specific types of leukocytes (Fig. 5). Indeed the name chemokine comes from a combination of *chemo*tactic and cyto*kines* and chemotaxis or signalling for directed migration has been the central concept, besides the structural similarities, that distinguishes this class of proteins. At the present time it is conceivable that the regulation of white blood cells' motility represents only a fraction of the spectrum of their functions.

Nevertheless, if many cytokines have been discovered by observation of their bioactivities, the majority of chemokines have been identified by cloning effort. In 1961 was described the first chemokine (PF4, platelet factor 4) and only in 1987 the second, IL-8 (67, 321), but as soon as few new molecules were identified and the common structural feature recognized, the number of chemokines increased rapidly.

Moreover since 1996, when it has been discovered that some chemokine receptors are able to bind HIV viruses, and serve as co-receptor for virus entry into the cell, chemokine research received a great and decisive impulse (58). Up to now more than 50 distinct chemokines are known, and their number is expected to increase significantly in the very near future, mainly as a result of the establishment and analysis of expressed sequence tag (EST) database.

Ranging in size from 6-14 kDa (non-glycosylated), chemokines are related by primary structure, particularly by the conservation of cysteine motifs. The superfamily is divided into four classes by the arrangement of these conserved cysteine (C) residues of the mature proteins:

- CXC chemokines or α-chemokines, have one amino acid residue separating the first two conserved cysteine residues;

- CC chemokines or β-chemokines have the first two conserved cysteines residues adjacent;

- C chemokines (γ-chemokines) lack two (the first and the third) of the four conserved cysteine present in CXC and CC chemokines;

- CX_3C chemokines, have three intervening amino acid residues between the first two conserved cysteine residues.

In the CC and CXC families, two disulfide bonds are present between the first and the third, and the second and fourth cysteine residues, respectively.

Within the α-subfamily, the chemokines can be further divided into two groups: the first having the characteristic three amino acid sequences ELR (glutamic acid-leucine-arginine) motif immediately preceding the first conserved cysteine residue near the amino terminus, and the second lacking the ELR motif.

Many of the ELR containing CXC chemokines have been show to be chemotactic for neutrophils, while non-ELR CXC chemokines act on lymphocytes.

By contrast, the CC chemokines have been found to be chemotactic for monocytes, lymphocytes, dendritic cells, natural killer cells, eosinophils and basophils, but not neutrophils. The unique γ-chemokine so far reported, lymphotactin, shows to be chemotactic for lymphocytes (148), while the single CX_3C chemokine (fractalkine), has been found to trigger the adhesion of T cells and monocytes. Fractalkine is a chemokine recently identified through bioinformatics, that unlike other known chemokines, is a type 1

membrane protein containing a chemokine domain at the amino terminus tethered on a long mucin-like stalk (16, 225).

Once secreted, most of chemokines are present in solutions in the dimeric as well as in the monomeric form. Some α- (IL-8, GRO-α, PF4) and β-chemokines (MIP-1β, RANTES, MCP-1), were analysed by either multidimensional NMR or X-ray crystallography (14, 175). As expected from the significant degree of sequence identity of these proteins, few are the significant differences in the structure at monomer level. When monomers form the dimeric protein, these differences play a significative role: the quaternary structure of α- and β-chemokines are entirely distinct and the dimer interface is formed by a completely different set of residues. While the dimer formed by α-chemokine monomers is globular, the one formed by β-chemokine monomers is elongated and cylindrical and these distinct quaternary structures are preserved throughout the two subfamilies. For some chemokines it was also reported the formation of tetramers comprising two dimeric subunits (270).

Although the biological significance of the dimeric form has yet to be established, the difference in the quaternary structures between α- and β-chemokines, suggests an explanation for the absence of receptor cross-binding between the two subfamilies (52). As above reported, CC and CXC chemokines act in a preferential way on different target cells and chemokine receptor-ligand interactions on inflammatory cells seem to be strictly regulated. Chemokines have two main sites of interaction with their receptors, one in the N-terminal region and the other within an exposed loop of the backbone that extends between the second and the third cysteine. Probably the receptor recognizes the loop region first, and this
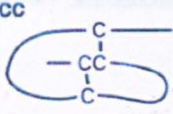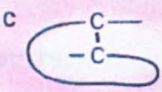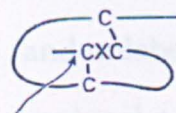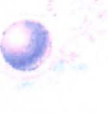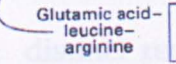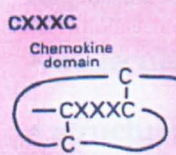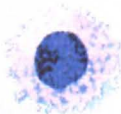
	Chemokine	Receptor	Cell Type
		Chemokine receptor	
	MCP-3, -4; MIP-1α; RANTES	CCR1	Eosinophil
	MCP-3, -4; eotaxin-1, -2; RANTES	CCR3	
	MCP-1, -2, -3, -4, -5	CCR2	Basophil
	MCP-3, -4; eotaxin-1, -2; RANTES	CCR3	
CC	MCP-3, -4; MIP-1α; RANTES	CCR1	
	MCP-1, -2, -3, -4, -5	CCR2	
	MIP-1α, MIP-1β, RANTES	CCR5	
	I-309	CCR8	Monocyte
	MDC, HCC-1, TECK	?	
	Fractalkine	CX₃CR1	
	SDF-1	CXCR4	
	MCP-3, -4; MIP-1α; RANTES	CCR1	
	MCP-1, -2, -3, -4, -5	CCR2	
	TARC	CCR4	
	MIP-1α, MIP-1β, RANTES	CCR5	Activated T cell
	MIP-3β (ELC)	CCR7	
	PARC, SLC, 6CKine (Exodus-2)	?	
	Fractalkine	CX₃CR1	
	IP-10, MIG, I-TAC	CXCR3	
C	PARC, DC-CK1	?	Resting T cell
	Lymphotactin	?	
	SDF-1	CXCR4	
CXC	MCP-3, -4; MIP-1α; RANTES	CCR1	
	MCP-1, -2, -3, -4, -5	CCR2	
	MCP-3, -4; eotaxin-1, -2; RANTES	CCR3	
	TARC	CCR4	Dendritic cell
	MIP-1α, MIP-1β, RANTES	CCR5	
	MIP-3α (LARC, Exodus-1)	CCR6	
	MDC, TECK	?	
	SDF-1	CXCR4	
	Interleukin-8, GCP-2	CXCR1	
Glutamic acid–leucine–arginine	Interleukin-8, GCP-2; GRO-α, -β, -γ; ENA-78; NAP-2; LIX	CXCR2	Neutrophil
CXXXC	MCP-1, -2, -3, -4, -5	CCR2	
	MIP-1α, MIP-1β, RANTES	CCR5	
	Fractalkine	CX₃CR1	Natural killer cell
	IP-10, MIG, I-TAC	CXCR3	

Figure 5. Chemokines and their Receptors.

Chemokines superfamily is composed by homologous 8-to-10kDa proteins that are subdivided into families on the basis of the relative position of the cysteine residues in the mature protein. In the a-chemokines, the first two cysteine residues are separated by a single aminoacid (CXC), whereas in the b-chemokines, the first two cysteine residues are adjacent to each other (CC). The C chemokine lymphotactin has only two cysteines in the mature protein, and the CXXXC chemokine fractaline has three amino acids separating the first two cysteines. Chemokine receptors are G-coupled proteins that are expressed on subgroups of leukocytes. At present four human CXC chemokine receptors (CXCXR1 trough CXCR4), eight human CC chemokine receptor (CCR1 trough CCR8) and one CXXXC chemokine receptor (CX3CR1) have been identified. In the figure are indicated the target cells on which chemokines display chemoattractant activity. Reproduction from: Luster A. D. Chemokines-Chemotactic Cytokines that mediate Inflammation. The New England Journal of Medicine. 388, 436-444.

interaction is necessary for the correct presentation of the triggering domain that correspond to the N-terminal binding site (264).

1.9 Chemokine Receptors

The recent findings of a large number of new chemokines and the efforts in understanding all their complex functions, leaded to an increased research interest for chemokine receptors.

Various approaches were used for the investigations of chemokine receptors:

- direct binding and displacement studies with radiolabelled ligands and unlabelled competitors;

- signal transduction studies attempting to predict the number of distinct receptors based upon Ca^{++} signal desensitization profiles in response to various chemokines;

- molecular cloning;

- structure function studies employing mutagenesis techniques.

At the present time, five receptors for CXC chemokines (CXCR) and eight receptors for CC chemokines (CCR) have been characterized (201, 237, 254).

All chemokine receptors are integral membrane glycoproteins (via N-linked glycosylation), with a heptahelical structure snaking through the membrane (7 Trans Membrane structure), the N-terminus on the extracellular face and the C-terminus on the cytoplasmic side of the membrane. Chemokine receptors, mainly expressed in immune cells and associated tissues, with a general average at 350 amino acids and a molecular

weight of approximately 40 kD, are smaller members of the rhodopsin-like, 100 G-protein coupled receptors family (4) (Fig. 6).

It is known that different G protein complexes exist which can bind to a given 7TM receptor. The 7TM receptor could be considered to be an adapter molecule which must discriminate between external ligands (the chemokines) and internal ligands (the G protein complexes) in order to transmit the appropriate signal to the cell (33, 63, 201).

When the signal is coupled to a $G\alpha_{i2}$ G-protein type, sensitive to *Bordetella pertussis* toxin, leads the signalling cascade to activation of phosphatidylinositol-specific phospholipase C, protein kinase C, small GTPases, Src-related tyrosine kinases, phosphatidylinositol-3-OH kinases and protein kinase B. Phospholipase C activates two second messengers: inositol-1,4,5-triphosphate, which releases Ca^{++} with a transient rise of the cytosolic Ca^{++} concentration, and diacylglicerol, which activates protein kinase C. Ca^{++} mobilization is determinant for superoxide production and cellular degranulation. Phosphatidylinositol-3-OH kinases can be activated also by the $\beta\gamma$ subunits of the G proteins, small GTPases or Src-related tyrosine kinases. The chemokine signalling circuit include also the activation of small GTP-binding proteins of the Ras, Rac and Rho families. Rac and Rho are involved into cytoskeletal rearrangements through regulation of actin-dependent processes and the consequential membrane ruffling and pseudopods formation that is required for cellular migration. In leukocytes, Rho family members relay signals from chemokine receptors also to cell-surface integrins, which trigger rapid adhesion before direct migration. Besides, the involvement of chemokine in cellular differentiation and proliferation, is due to the ability of G protein-coupled receptors to activate,
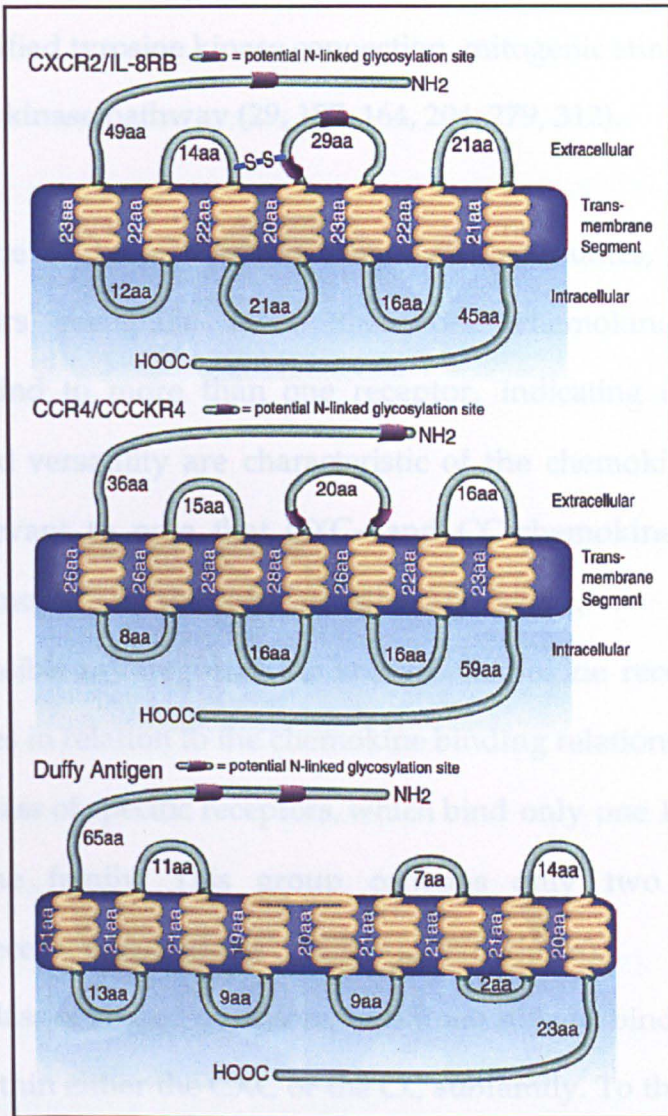
Figure 6. Chemokine Receptors.
Chemokines mediate their activities by binding to target cell surface chemokine receptors that belong to the large family of G protein-coupled, seven transmembrane (7 TM) domain receptors. Based on the receptor nomenclature established at the 1996 Gordon Research Conference on chemotactic cytokines, the chemokine receptors that bind C-X-C chemokines are designated CXCRs and the receptors that bind C-C chemokines are designated CCRs. In addition, the Duffy blood group antigen (DARC) has been shown to be an erythrocyte chemokine receptor that can bind selected C-X-C, as well as C-C chemokines. Leukocytes have generally been found to express more than one receptor type. The various CXCRs and CCRs are known to exhibit overlapping ligand specificities.

via an unidentified tyrosine kinase connection, mitogenic stimuli through the Ras/Raf/Map kinase pathway (29, 157, 164, 204, 279, 312).

From the evidences derived from different studies, it emerges that most receptors recognize more than one chemokine, and several chemokines bind to more than one receptor, indicating once more that complexity and versatility are characteristic of the chemokine system (237, 254). It is relevant to note that CXC- and CC-chemokine receptor only recognize chemokines of the corresponding subfamily.

It is possible to categorize the known chemokine receptors into four separate classes in relation to the chemokine binding relationship .

• The class of specific receptors, which bind only one known ligand of the chemokine family. This group contains only two examples: the CXCR1(IL-8 receptor A), and the CXCR4 (LESTR/fusin).

• The class of shared receptors, which are able to bind more than one chemokine within either the CXC or the CC subfamily. To this group belong the largest number of known receptors to date. The CXC2 receptor (IL-8 receptor B) binds to all E-L-R CXC chemokines described, while CXCR3 binds to CXC chemokines in which the E-L-R domain is not present. All the CC receptors (CCR1-CCR8) known belong to this group.

• The Duffy blood group antigen (DARC), originally described as an erythrocyte chemokine receptor, is the only promiscuous receptor characterized by binding properties to chemokine ligands either CXC or CC branches (209).

• Virally encoded chemokine receptors represent shared receptors that have been transduced into the viral genomes during evolutionary

history. At present time two receptors are described: a CC receptor encoded by a cytomegalovirus open reading frame CMV US28 , and a CXC receptor encoded by Herpes Saimiri virus open reading frame (4, 233).

There are some receptors homologous to chemokine receptors for which no ligands have yet been identified (orphan receptors). Similarly there are new chemokines, such as C10, of which their receptors are also unknown.

The presences of receptor mRNA and immunodetectable receptor protein in the membrane fraction are considered to be acceptable evidence for a cell type to be a specific target for a given chemokine.

CXCR1 and CXCR2 are expressed by neutrophils but not by B and T lymphocytes, while CXCR3 is highly expressed by IL-2-activated T lymphocytes (132, 176, 202). CXCR4 (LESTR/fusin), was originally discovered as an orphan receptor with structural similarity to chemokine receptors; subsequently it was identified as a necessary cofactor for entry of T cell-tropic HIV virus into CD4^{+} cells, and the chemokine PBSF/SDF-1 was described as its ligand(27, 85, 213).

The CCR1 (MIP-1α/RANTES receptor) is expressed on monocytes, neutrophils or eosinophils, while CCR2A and CCR2B are expressed only on monocytes (47, 211, 314). CCR3 is the receptor for eotaxin, a chemokine molecule that act specifically on eosinophils (235). CCR4, CCR6 and CCR7 can be expressed by T lymphocytes, depending on the state of cell activation, and by B lymphocytes. The ligands of these molecules, respectively TARC, LARC and ELC, are chemokines expressed constitutively in lymphoid tissues (lymphnodes, thymus, Peyer's patches, T cell areas of the spleen) and their

apparent selectively for receptors expressed by lymphocytes suggest that are involved in the regulation of physiological lymphocyte traffic (320).

CCR5 mediates the activity of MIP-1α, MIP-1β and RANTES in primary adherent monocytes, while is not expressed in neutrophils and eosinophils. Recently it has also been shown that CCR5, as well as CXCR4, plays a critical role in HIV virus entry into the target CD4$^+$ cells. CXCR4, as reported above is expressed in CD4$^+$ T cells, while CCR5 is described to be the co-receptor for macrophage-tropic HIV-1 virus. This finding is consistent with the report identifying the CCR5 ligands (MIP-1α, MIP-1β and RANTES), as the major suppressive factors produced by CD8+ T cells for macrophage-tropic, but not T cell tropic, HIV isolates (28, 53). Studies on individuals repeatedly exposed to HIV but remaining uninfected, revealed a homozygous defect in their CCR5 gene, leading to the expression of a truncated CCR5 molecule, undetectable on cell surfaces. In these individuals the LESTR/fusin receptor have been shown to be unaltered and the CD4$^+$ T cells to be easily infected by T cell-tropic viruses (65). All these data suggest that macrophage-tropic isolates are likely to be the viruses responsible for the transmission of HIV-1 viruses and that CCR5 is a determinant factor in the transmission and progression of AIDS (Fig. 7).

1.10 Gene location

Genes for many CXC and CC chemokines have been found to be clustered. Most of the CXC chemokines genes have been mapped to human chromosome 4q, and those for many CC chemokines on human chromosome 17q (mouse chromosome 11). Exceptions to these cluster

Figure 7. Chemokine Receptors as Obligate Coreceptors for HIV Entry into Cells and Chemokine Inhibition of HIV Entry.

HIV glycoprotein 120 (gp120) binds to CD4, resulting in a conformational change that expose the V3 loop in gp120 and permits subsequent interaction with a chemokine receptor. To gain entry into cells, macrophage tropic (M-tropic) HIV-1 uses CCR5 predominantly, and the T-cell-tropic (T-tropic) HIV-1 uses CXCR4 predominantly. Macrophage inflammatory proteins (MIP) -1α and -1β and the RANTES (regulated upon activation normal T-cell expressed and secreted) chemokine, ligands for CCR5, block M-tropic HIV-1 from entering cells. Stromal-cell-derived factor 1 (SDF-1), a ligand for CXCR4, blocks T-tropic HIV-1 from entering cells.

Reproduction from: Luster A. D. Chemokines-Chemotactic Cytokines that mediate Inflammation. The New England Journal of Medicine. 388, 436-444.

includes some CXC and CC molecules, nevertheless the clusters of chemokine genes suggest that many chemokine family members arose through gene duplication and subsequent divergence. The gene for lymphotactin has been localized on chromosome 1 both in human and mouse, while that for fractalkine on human chromosome 16q and mouse chromosome 8 (288).

1.11 General function of Chemokines

A large number of information about chemokines and their functions arose from the recent progress in this field and demonstrates the complexity of this cytokine subfamily. Chemokines are produced by a variety of cell types and have overlapping activity. In certain cases peculiar functions, as the inhibition and stimulation of blood vessel formation (angiogenesis) (153) or leukocyte maturation in the bone marrow (myelopoiesis) (37, 293), were attributed to chemokines, but most chemokine actions that have been reported are related to leukocyte migration and recruitment. (255, 269) (Fig. 8).

The circulatory and migratory properties of leukocytes have evolved to allow efficient surveillance of tissues for infectious pathogens and rapid accumulation at sites of injury and infection. Lymphocytes, in particular, continuously patrol the body for foreign antigens recirculating from blood, through tissue, into lymphatic vessels, and back to blood (42).

The *in vivo* requirements for cell trafficking are complicated and need a coordinate sequence of events:
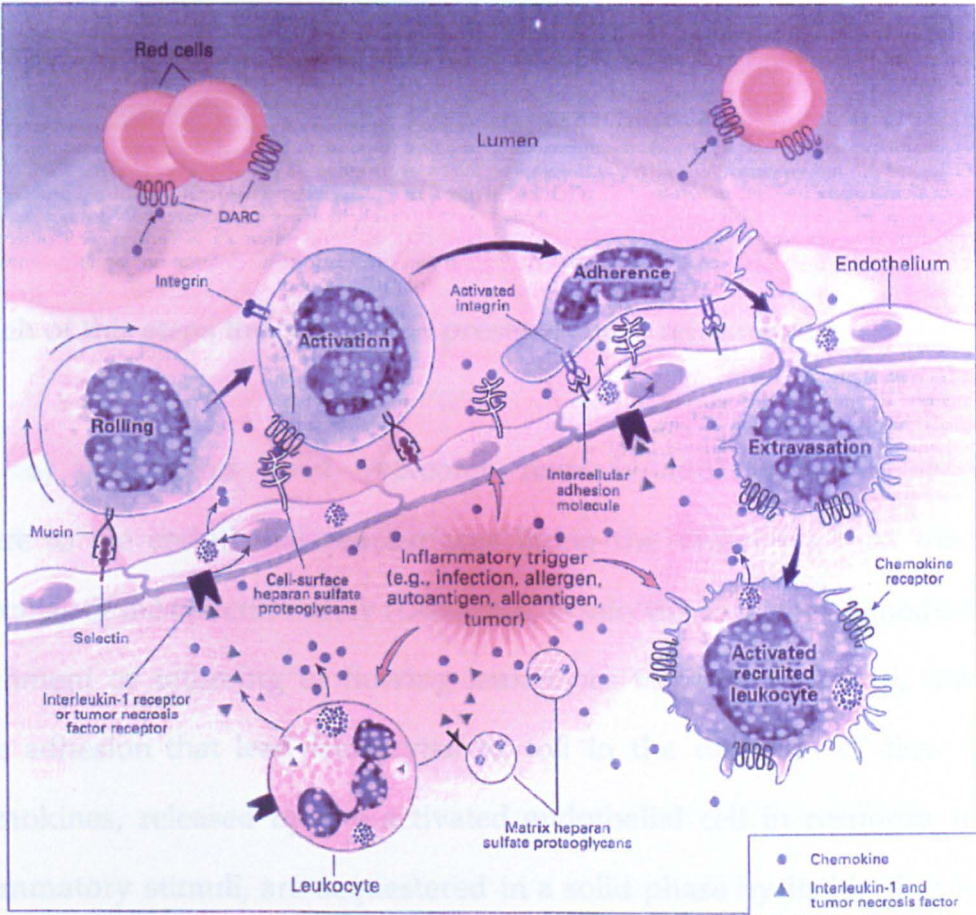
Figure 8. Chemokine Regulation of Leukocyte Trafficking.
Chemokines are secreted at sites of inflammation and infection by resident tissue cells, resident and recruited leukocytes, and cytokine-activated endothelial cells. Chemokines are locally retained on matrix and cell-surface heparan sulphate proteoglycans, establishing a chemokine concentration gradient surrounding the inflammmatory stimulus, as well as on the surface of the overlying endothelium. Leukocytes rolling on the endothelium in a selectin-mediated process are brought into contact with chemokines retained on cell-surface heparan sulphate proteoglycans. Chemokines signalling activates leukocytes integrins, leading to firm adherence and extravasion. The recruited leukocytes are activated by local proinflammatory cytokines and may become desensitized to further chemokine signaling because of high local concentrations of chemokines. The Duffy antigen receptor for chemokines (DARC), a non signaling erythrocyte chemokine receptor, functions as a sink, removing chemokines from the circulation and thus helping to maintain a tissue-bloodstream chemokine gradient.

a) circulation

b) adhesion

c) diapedesis

d) migration.

In each of this steps the chemokine presence has a relevant role.

a) Leukocytes must overcome hemodynamic forces in order to adhere to the endothelial cell surfaces lining the vessel wall. At this step molecules of the selectin family (L-selectin, P-selectin, E-selectin), mediate the attachment or tethering of flowing leukocytes to the vessel wall, through labile adhesion that lead leukocytes to roll in the direction of flow (269). Chemokines, released by the activated endothelial cell in response to the inflammatory stimuli, are sequestered in a solid phase by its binding to cell surface proteoglycans or by association with heparin (280) and form chemical gradients. Such immobilization allows them to be presented to the marginating leukocytes and to convert the initial rolling interaction of leukocytes in a stable binding to surface integrin adhesion molecules of the endothelial cell.

b) Specific chemokine receptors present on the target leukocyte interact with chemokines inducing a rapid accumulations of intracellular free calcium (267). Calcium mobilization is a critical intracellular event that not only stimulate degranulation, shape change, actin polymerization and respiratory burst (289), but also regulate the expression of adhesion molecules on leukocyte surfaces. Chemokines upregulate adhesiveness

through integrins by inducing fusion of secretory granules with the plasma membrane of activated cells. The expression of molecules such as MAC-1 (CD11b/CD18), LFA-1, p150,95 on the surface of leukocytes, enable these cells to adhere to the endothelium integrin ligands (ICAM-1, ICAM-2, ICAM3), before migrating into the tissues (269).

c) Changes in cell shape following chemokine stimulation is due to a dynamic remodelling of the cytoskeleton with the formation of cytoplasmic projections termed "uropods" or "lamellipodia". This morphological modification allows adherent leukocytes to cross the endothelial lining (extravasion or diapedesis), and pass into the tissue space.

d) Once leukocytes have crossed the vascular endothelium into the tissue their migration to the focus of stimuli is directed by the gradient of chemokines molecules present within the extracellular matrix.

Over the past years the role of chemokines, in addition to the one described in infection and in inflammation, it has been also recognized their participation in the regulation of lymphocyte traffic. T and B lymphocytes, during their development and differentiation move through non-lymphoid and lymphoid tissues (Fig. 9). Several novel lymphocyte-selective chemokines, that are expressed constitutively at high levels in the thymus, lymphnodes and other lymphoid tissues have been identified (123, 136, 248, 318). Most of these chemokines (TARC, ELC, 6Ckine, and LARC) belong to the CC subfamily and it is conceivable that may be involved in driving the trafficking of T lymphocytes.
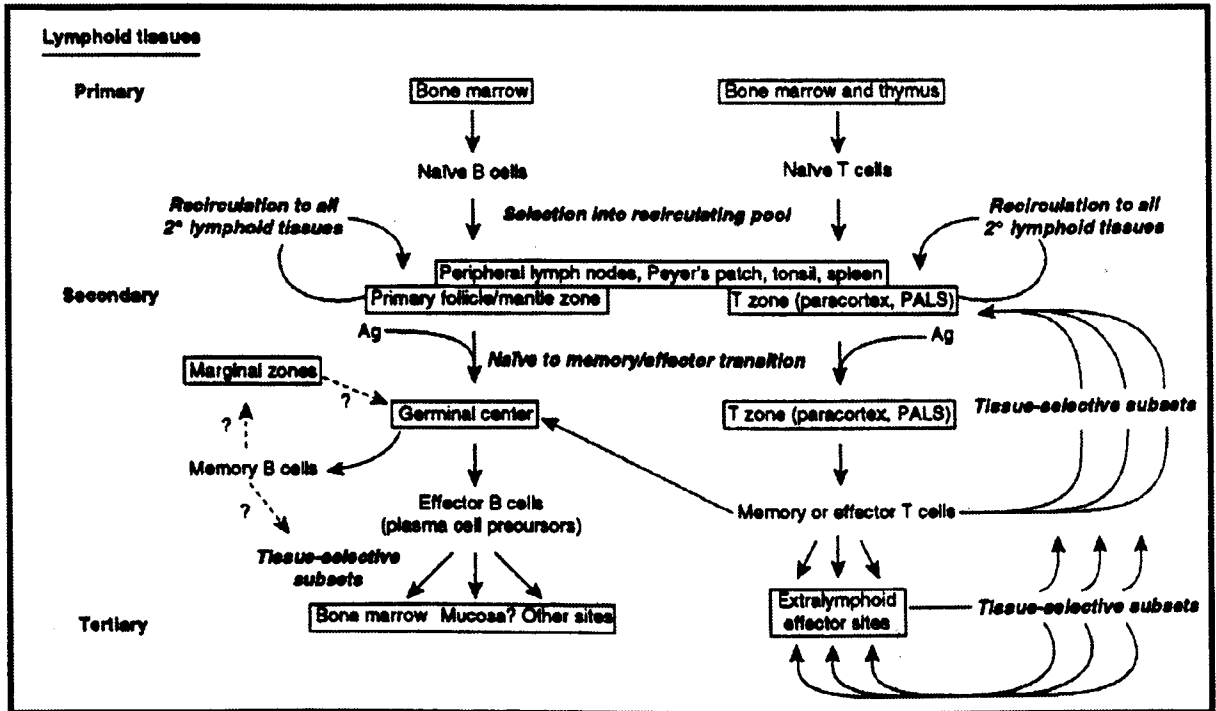
Figure 9. Lymphocytes Homing and Trafficking.

Naive lymphocytes home to specific microenvironments within secondary lymphoid tissues and recirculate through these sites until they either die or encounter their specific antigen. Unlike naive cells, memory and effector T (and probably B) cells can efficiently extravasate in tertiary (extralymphoid) inflammatory sites, with subset displaying targeted trafficking through, for example, inflamed skin, intestinal mucosa, pulmonary tissues and joints. Antigen activated B cells may home to specialized environments in the outer T zone during primary responses, or may colonize germinal center sites of hypermutation, affinity maturation, and cell memory differentiation. Less abundant, specialized lymphocytes subset include γδ T cells in the mouse, and subset of gut intraepithelial leukocytes. That may be target directly from their origin in the thymus or bone marrow to reproductive, cutaneous, intestinal or other tertiary tissues (not illustrated). The extralymphoid effector sites of selective homing include (at least) skin, lung, intestinal lamina propria, and synovium. PALS, Periarticolar lymphoid sheath. Ag, antigen.

The specific expression of DC-DK1 by dendritic cells at the site of initiation of an immune response, combined with its chemotactic activity for naive T cells (3), suggests that chemokines like DC-DK1, play an important role in the induction of immune responses.

BCL a new CXC chemokine that is secreted by lymphoid tissue cells and selectively bind to the CXCR5 receptor (BLR1) expressed in B lymphocytes, is another reported example of a tissue specific function of the chemokines (115). Finally, an abnormally low number of B-lymphocytes and a severely defective lymphopoiesis, was reported in mice lacking the SDF-1 gene, suggesting that this chemokine, previously described as growth factors for B-lymphocytes progenitors, may be involved in directing progenitor cells into the appropriate maturation sites in the bone marrow (205).

1.12 Other chemokines activities

Not all of the known properties of the chemokines involve leukocytes migration; here it will be briefly reported other non-chemotactic properties of chemokines.

- Haemopoiesis

SDF-1 (for Stromal Derived Factor 1), in the two forms (α and β) generated by alternative splicing, is a CXC chemokine constitutively expressed in a broad range of tissues. As above mentioned SDF-1 is involved in B-lymphocytes maturation, but more generally this chemokine was described to support the colonization of the bone marrow by haemopoietic precursors during embryogenesis (5).

Another CXC chemokine, platelet factor 4 (PF4), has been reported to have a large spectrum of activities, including, differently from SDF-1, an inhibitory effect on haemopoietic processes (106). Inhibition of megakaryopoiesis, as well as suppression of colony formation by immature myeloid progenitors after GM-CSF stimulation, has been documented.

The MIP proteins (CC chemokine subfamily), have been reported to have some interesting effects on haemopoietic precursor cells. MIP-1 and murine MIP-2, synergistically with GM- and M-CSF, are able to enhance CFU-GM. MIP-1α in the monomeric form reversibly inhibits the proliferation of stem cells, but this suppressive effect does not take place when the chemokine is in the dimeric form (38, 110).

• Pyrogenic effects

To the MIP-1 proteins have been ascribed also pyrogenic activities. The pharmacological aspects of this activity have been investigated and the evidence from these studies suggest that MIP-1 exerts its febrile effects by some direct interaction with the hypothalamus (194).

• Chemokines in Tumour Biology

In recent years some experiments have been performed in order to establish if chemokines secretion can be involved in inhibition of tumour growth *in vivo*. Tumour derived cells, which are normally tumorigenic in nude mice, were engineered to produce different CC chemokines such as MCP-1 and RANTES and were inoculated into the animal model. In all cases the chemokine-bearing tumour cells failed to grow, while the unmodified cells formed large tumours (247). The observations that a number of monocytes and macrophages were associated with the injection site and that the co-administration of antibodies, which inhibited T-cell function or

macrophage migration, rescued the tumour growth, confirmed that the inhibition of tumour growth was an immune-mediated phenomenon induced by the chemokines (199). These data suggested a possible use of chemokines in anti-tumour strategies. However these results were not in agreement with different finding that many tumour cell as well as primary tumours are producers of chemokines

Whether tumour cells may derive any advantage from chemokines production, is not yet clearly determined yet, but an involvement of chemokines in beginning the metastatic process by drawing the migration of tumoral cells into vessels, and the angiogenetic properties of some of these, has been suggested by several authors to play an important role (111).

Finally, a CXC chemokine referred as MGSA (Melanocyte growth stimulatory activity), or GRO–α was described as an autocrine growth factor for a human melanoma cell line (242).

From these findings it is evident that chemokines' role in tumour biology is still not well understood, but the increasing interest in their therapeutic potentials will certainly lead to a better defined overall picture.

• <u>Angiogenesis</u>

Endothelial cells produces various chemokines in response to signals representative of inflammatory reactions, immunity and thrombosis (184). Furthermore recent evidences suggest that some chemokines may affect endothelial cells functions. IL-8, GRO-α, and other CXC chemokines were reported to induce endothelial cells migration and proliferation *in vitro* and to be angiogenic *in vivo* (153). PF4 and IP-10 (Interferon γ-inducible protein 10), were shown to have angiostatic properties *in vivo* (295). As described above, within the CXC subfamily, it is possible to identify a group of

molecules in which the ELR (glutammic acid-leucine-arginine) motif is highly conserved, and a second lacking such an ELR motif. Recent result suggest that the presence or absence of an ELR motif dictate whether CXC chemokines induce or inhibit angiogenesis.

There are no findings that CC chemokines affect endothelial cells migration and proliferation.

• <u>Chemokines in Disease</u>

While inflammation and leukocyte accumulation is essential to innate host defence against microbes, the presence of infiltrating leukocytes in non-infectious disorders may be deleterious to the host, leading to impaired organ function and potential mortality.

It is at present established that certain CC chemokines (RANTES, MCP-1, MCP-3, eotaxin), exhibit potent promigratory and activating potentials for basophils, eosinophils and T cells, the cells most often associated with respiratory pathologies and allergic disorders (14, 178, 180, 235) (Fig. 10).

Rheumatoid arthritis (RA) is an autoimmune disease characterized by joint inflammation and destruction that is related to the sequestration of leukocytes within the developing pannus and synovial space. A number of studies have determined that both CXC and CC chemokines are significantly present in RA, even if their involvement in the pathogenesis of this disorders is still unclear (152). Recent data demonstrate that MIP-1α and MIP-2 contribute to the development of experimental arthritis in mice (146), and this finding suggest that these chemokines may play a similar role in the pathogenesis of human RA.

Inflammatory Disease	Infiltrate	Chemokine
Acute respiratory distress syndrome	Neutrophil	Interleukin-8; GRO-α, -β, -γ; ENA-78
Asthma	Eosinophil, T cell, monocyte, basophil	MCP-1, -4; MIP-1α; eotaxin; RANTES
Bacterial pneumonia	Neutrophil	Interleukin-8; ENA-78
Sarcoidosis	T cell, monocyte	IP-10
Glomerulonephritis	Monocyte, T cell, neutrophil	MCP-1, RANTES, IP-10
Rheumatoid arthritis	Monocyte, neutrophil	MIP-1α, MCP-1, interleukin-8, ENA-78
Osteoarthritis		MIP-1β
Atherosclerosis	T cell, monocyte	MCP-1, -4; IP-10
Inflammatory bowel disease	Monocyte, neutrophil, T cell, eosinophil	MCP-1, MIP-1α, eotaxin, IP-10, interleukin-8
Psoriasis	T cell, neutrophil	MCP-1, IP-10, MIG, GRO-β, interleukin-8
Bacterial meningitis	Neutrophil, monocyte	Interleukin-8; GRO-α; MCP-1; MIP-1α, -1β
Viral meningitis	T cell, monocyte	MCP-1, IP-10

Figure 10. Role of Chemokines in Various Inflammatory Disease. Inflammatory disease are characterized by selective accumulation of leukocytes subgroups, a process controlled by the expression of certain chemokines. Each disease has a characteristic inflammatory infiltrate in which chemokine messenger RNA or protein concentrations have been shown to be up-regulated.

Reproduction from: Luster A. D. Chemokines-Chemotactic Cytokines that mediate Inflammation. The New England Journal of Medicine. 388, 436-444.

Finally, ischemia/reperfusion injury contributes to the pathophysiology of many clinical disorders, including myocardial infarction, stoke, mesenteric ischemia, peripheral vascular disease, organ transplantation and circulatory shock.

Investigations have shown that anoxia/hyperoxia (simulating ischemia/reperfusion) can lead to a significant production of IL-8 by mononuclear and vascular pulmonary endothelial cells (145, 190). The high level of IL-8 expression correlates with a relevant neutrophils extravasion and tissue injury, that are prevented by immunization of the animal models with neutralizing Abs to IL-8 (259).

1.13 α-Chemokines

• KC: This protein is the ELR-CXC murine chemokine homologous of the human MGSA (Melanoma Growth Stimulatory Activity)/ GRO-α chemokine.

The KC gene is localized on chromosome 5 and its mRNA is 892 bases long with a cds that encodes a precursor protein of 96 amino acids (10.236 daltons) (218, 288).

The 72 amino acids mature KC protein is secreted by different cell types (fibroblast, macrophages, endothelial cells), after induction of agents associated with wound healing and inflammatory response such as serum, PDGF, γ-interferon and bacterial enterotoxins. KC, binding to the IL-8R type B homolog specific receptor, induces chemotaxis, respiratory burst and overexpression of CD11b/ CD18 in neutrophil (107, 198).

Although the high sequence similarity between the human MGSA/gro α gene and the mouse KC gene (65%), the transcriptional control of the gene is different (214) and the mitogenic activity on melanocytes reported for MGSA, was not described for the mouse chemokine.

•MIP-2: Macrophage Inflammatory protein-2 α and β are the products of the GRO-β and GRO-γ genes described in humans, in mouse a single MIP-2 chemokine was reported. The murine MIP-2 genomic clone displays the canonical four exon/three introns structure typical of other genes in the chemokine α subfamily. The promoter region contains an LPS-responsive element in the region that contains a conserved NF-kB consensus motif highly conserved in all three human GRO genes and described also in the mouse KC gene. MIP-2 RNA has 1008 bp with an open reading frame of 300 bp, which encodes for a 100 amino acids precursor protein. The mature MIP-2 has 73 amino acids.

Several inflammatory stimuli induce the rapid and marked increase in MIP-2 secretion by mast and mesangial cells, alveolar macrophages and epidermal cells (313) with a rapid and evident effect on neutrophils and basophils. Probably MIP-2 and KC chemokines provide in mouse the same functions of IL-8, the major neutrophil chemoattractant in humans, that in mouse has not been identified. In order to establish MIP-2 contribution in different disorders, many studies were performed on animals (84, 302). MIP-2 binds with high affinity to murine IL-8 RB homologue (278).

•CRG-2/C7: The product of this gene is the murine homologue of the human gamma interferon inducible protein-10 (IP-10). The gene has the

above described classical organization. The mature protein, which lack the ELR domain, is formed by 77 amino acids and binds to epithelial, endothelial and haematopoietic cells via a specific IP-10/Mig receptor (CXCR3) (83). The CXCR3 is highly expressed in IL-2 activated T lymphocytes and this finding suggest that IP-10 (CRG-2) is involved in the selective recruitment of effector T cells (177). IP-10 act also on monocytes and NK cells, but not neutrophils. Cytokines, growth factors, bacteria products, viruses and other stimuli induce IP-10 chemokine secretion by endothelial cells, fibroblasts and monocytes, but relevant is the presence of spontaneous high levels of IP-10 transcripts found in lymphoid organs (spleen, thymus, lymph nodes) (103, 281).

Several investigations were performed to evaluate the IP-10 role in animal model of inflammatory related disease (288).

•Mig-1: Interferon-gamma induces in monocytes and macrophages the expression of Mig-1 (monokine induced by interferon-gamma), a CXC chemokine active as chemoattractant for activated T cells. This molecule is functionally related to IP-10, with which is shares a receptor, CXCR3. The RNA open reading frame encodes for a 126 amino acids precursor protein, while the mature form has 105 amino acids (82, 83).

• BCL: B-lymphocytes chemoattractant is a novel chemokine, that is highly expressed in the follicles of Peyer's patches, spleen and lymph nodes. BCL strongly attracts B lymphocytes while promoting migration of only a small numbers of T cells and macrophages. It is the first chemokine to be identified that is selective towards B cells. BCL binds to the orphan

chemokine receptor BLR-1 (Burkitt's lymphoma receptor 1), acting to direct the migration of B lymphocytes to follicles in secondary lymphoid organs. The 1.16 kb RNA encodes for a 109 amino acids proteins (116).

1.14 β-Chemokines

• JE: is the murine homologue to human MCP-1 (Monocyte chemoattractant protein-1), a CC chemokine that plays an important role in chronic inflammatory disease controlled by mononuclear leukocytes. After stimulation with different cytokine or cytokine inducers (bacterial enterotoxins, growth factors, viruses, LPS), MCP-1 (JE), is secreted by various cells types, including fibroblasts, epithelial cells, leukocytes, keratinocytes, endothelial cells, smooth muscle cells, as well as tumour cells (254). MCP-1 is major chemoattractant for monocytes, T lymphocytes, dendritic cells and basophils, in which induces also a histamine release (14, 24, 45). The murine JE gene is located on the distal portion of chromosome 11 and it contains three exons; the RNA when spliced is either 594 or 797 bases and encodes for a 148 amino acids precursor protein that matures in the 125 amino acids form. Like others CC chemokines, MCP-1 binds to the shared receptors CCR1, CCR2, DARC and to the virally encoded US28 (159).

MCP-1 (JE) is a prototypic β–subfamily chemokine and over the past years was extensively studied for its expression and contribution in humans and animals disease (288).

• RANTES: (Regulated on Activation, Normal T-cell Expressed and Secreted), is a chemokine originally identified by molecular cloning as a

transcript expressed in T, but not in B lymphocytes. A different name, TY5, is also used to define the murine homologue. It is the only β–chemokine present in platelets, and shows a potent chemotactic and activating properties for basophils, eosinophils, NK and dendritic cells. The murine RANTES is the product of the *Scya5* gene that is located on chromosome 11, and is organized into three exons and two introns; the mRNA encodes for a protein that in the mature form display 68 amino acids. The expression of RANTES is induced by cytokines (IL-1, TNF-α, IFN-γ), thrombin, PMA or PHA into several cell types like T lymphocytes, epithelial cells, fibroblasts, mesangial cells and some tumour cells. RANTES binds to the CCR1, CCR3, CCR5, DARC and US28 receptors inducing chemotaxis, but also expression of adhesion molecules, enzymes release or degranulation into target cells (142, 237, 249). RANTES expression was described in several human diseases as rheumatoid arthritis, in acute renal allograft rejection and in asthmatics and allergic subjects (14, 275).

Finally, it was observed a HIV-suppressive effect of RANTES that synergize with MIP-1α and MIP-1β in competition with macrophage-tropic HIV isolates for CCR5 binding (53).

•MIP-α and MIP-β: Macrophage inflammatory protein alpha and beta are members of the CC chemokines subfamily that share 70% homology; the murine counterparts are respectively the SISα and the H-400 molecules. Despite the structural similarity, MIP-1α and MIP-1β show significant differences in their functions. MIP-1β is inactive, unlike MIP-1α, in the activation of neutrophils. MIP-1α inhibits early haemapoietic progenitor growth, whereas MIP-1β potentiates it. In terms of HIV-suppressive effects,

both molecules seem to have a synergistic effect (53). Secretion of both molecules is induced by several inflammatory stimula (cytokines, bacterial products and others), in a wide range of cell types like fibroblasts, lymphocytes, monocytes, smooth muscle cells.

Both murine MIP-1α and MIP-1β genes are located on the distal portion of the chromosome 11 near the *Hox-2* gene complex, and display the same structure with three exons and two introns. The mature secreted forms have 68 and 69 amino acids. MIP-1α and MIP-1β bind to the same receptors (CCR1, CCR5, US28), with the exception of CCR4 to which bind only MIP-1α (208, 300).

Both molecules are extensively studied, and a number of data are available about their regulation of expression, the expression in several disease (in humans and in animal models), and biological effects in vivo and in vitro (254, 288)

•6Ckine/Exodus-2/SLC: 6Ckine, also named SLC (Secondary lymphoid-tissue chemokine) and Exodus-2, is a CC chemokine recently discovered independently from the EST database by three groups. 6Ckine contain the four conserved cystein characteristic of β-chemokines plus two additional cystein in its long carboxyl-terminal domain. Mouse cDNA encodes a 133 amino acids protein with a 23 residue signal peptide that is cleaved to generate the 110 amino acids mature protein. This chemokine is highly expressed in lymphoid tissues such as lymph nodes, spleen, appendix and (only in mouse) lung. Unlike most CC chemokines, 6Ckine is not chemotactic for monocytes, macrophages and B cells, but acts on thymocytes

and *in vitro* activates T cells. Recently was reported that 6Ckine/SLC is an agonist for the MIP-3β receptor CCR7 (43, 123).

•C10: this murine chemokine belongs to the Macrophage Inflammatory Protein (MIP)-related protein-2 (MRP-2) subfamily, and only recently has been isolated a novel human C-C chemokine (MIP-1 delta), that probably corresponds to the human homologue (301). C10 transcripts were identified in bone marrow cells, myeloid cell lines, T lymphocytes and macrophages. Although C10 is a CC chemokine, the regulation of its expression differs from the one reported for other molecules of the same subfamily. MIP-1α, RANTES, JE are all induced by LPS treatment of bone marrow-derived macrophages (BMM) and/or resident peritoneal macrophages (RPM), LPS stimulation of C10 was never observed. Conversely IL-3 and GM-CSF strongly induce C10 in both macrophage populations, whereas MIP-1a, RANTES and JE show only a weak induction restricted to BMM. Finally was reported that macrophage stimulation by IL-4 induce a selective enhancement of C10 secretion, but fail to stimulate any of the other CC chemokines (221, 222). These data suggest that C10 may have distinct functions in host defence, but at present its biological significance is not know.

Linkage studies showed that the C10 genes (*Scya6*) is closely linked to the *Scya2* locus on mouse chromosome 11, as other members of the beta-chemokine family, but in contrast with the common three-exon genomic structure, C10 contains a novel second exon that was not previously described (23). The precursor protein shows 116 amino acid residues, of

which 21 belong to the signal peptide. To date the C10 binding receptor is still unknown.

1.15 γ-Chemokine

• <u>Lymphotactin:</u> is the only example of the γ-chemokine subfamily, it shows similarity to some members of the β-chemokine, but it lacks the first and third cysteine residues characteristic of the others chemokines. The murine *Ltn* gene maps in the distal region of chromosome one; the mRNA, of approximately 0.9kb, is exclusively expressed in activated thymic CD8$^+$ CD3$^+$ cells and CD8$^+$ CD3$^+$ T cells derived from the spleen. Functional analysis demonstrate that lymphotactin is chemotactic for lymphocytes but not for monocytes, a characteristic that makes it unique among chemokines. The structural features predict the cleavage and secretion of a mature Ltn protein of approximately 10 kD. Lymphotactin bind to XCR1 a recently described receptor (148, 149, 319).

Chapter 2

Materials and Methods

2.1 Animals and Tumour Induction

Animals of Balb/c, CBA, Swiss and ASN strains were obtained from JacKson Laboratories, Milano, Italy and M.B.S., Treviso, Italy. For tumour induction, animals were injected twice, at a 15 days interval, intraperitoneally with 200µl of emusifield (1:1 with PBS) incomplete Freund's Adjuvant. A single adjuvant injection or pristain oil (Sigma, ST. Luis, Missoury, U.S.A.), following the standard protocol, and PBS as control, were also evaluated for tumour induction. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international policies (EEC Council Directive 86/609, OJ L385,1, Dec.12 1987; NIH Guide for the care and use of Laboratory Animals, NIH Publication No. 85-23, 1985).

2.2 Histology and Electron Microscopy

After two weeks of the second injection mice were sacrificed. Tumours were surgically removed and fixed in 10% formalin in PBS and paraffin embedded. Sections of 4-6 µm thickness were cut and stained with

haematoxylin/eosin. Sudan III staining (ICN Biomedicals S.r.l., Milano, Italy) was performed on cryostat sections of lymphangioma following the standard method. Sections were examined at the optical microscope and photographed. For electronmicroscopy, tumour samples were fixed with 2.5% glutaraldehyde in 0.1M sodium phosphate buffer, pH 7.3 for 1 hour at 4°C. Post-fixation was performed in 1% osmium tetroxide (Sigma) in 0.1 M cacodylate buffer, pH 7.3. Specimens were dehydrated up to 100% ethanol, treated with propylene-oxide and embedded in Araldite (Fluka, Switzerland). Polymerization was carried out at 60°C for 3 days. Ultrathin sections were cut with an Ultracut ultramicrotome (Reichert Jung, Germany). Grids were stained with uranyl acetate and lead citrate, and examined with a JEOL-JEM 100S transmission electron microscope. Histological specimens were also obtained from Araldite embedded samples. Semithin sections of 1 nm thick were cut with the ultramicrotome and stained with 1% toluidine blue in 0.5% sodium carbonate at 90°C, and observed with a Zeiss Axiophot microscope.

The preparation of the histological sections was done in collaboration with Prof. Giorgio Stanta of the Istituto di Anatomia Patologica, Università degli Studi di Trieste. The electron microscopy specimens were done by Dr. Marina Zweyer of the Dipartimento di Morfologia Umana Normale, Università degli Studi di Trieste.

2.3 Immunohistochemistry

Lymphangioma fragments were frozen in liquid nitrogen and stored at -20°C; 8-10 μm cryostat sections were fixed in acetone and

immunostaining was performed as described (291). Sections were stained with the rat anti-mouse CD31/PECAM mAb Mec 7.46 (kindly provided by A. Vecchi, Istituto di Ricerche Farmacologiche Mario Negri, Milano, Italy), a biotinylated rat anti-mouse ICAM-2 mAb (Pharmingen), and a biotinylated rat anti-mouse ICAM-1/CD54 mAb (Jackson, West Grove, PA, U.S.A.). Anti-Flt-4 and anti-Flk-1 were affinity-purified rabbit polyclonal antibodies (Santa Cruz). As second antibody, a biotin-conjugated goat anti-rat (Vector Laboratories) or a biotin-conjugated anti-rabbit IgG (Dako) was used. Reactions were developed with Fast Red (Sigma) after incubation with alkaline-phosphatase-conjugated Streptavidin (Boehringer Mannheim).

2.4 Preparation of Pituitary extract (Endothelial Growth Factor)

All the procedure was carried out at 4°C. The hypothalamus area was excised from fresh bovine brain, washed in NaCl 0.1M and homogenized (1Kg of hypothalamus/1.25 l NaCl). The mixture, pH of 6.8, was kept in agitation for at least 2h. After centrifugation (13800g 40min.) the supernatant was collected and streptomycin sulphate (Sigma) (0.5 gr./100 ml) was added. The mixture was kept in agitation overnight, centrifuged (13800g 40min.) and aliquoted in eppendorf tubes. Storage was done at -80 4°C. Different dilutions of the pituitary extract were tested on HUVEC cells and routinely used 1:200 dilution in fresh medium.

2.5 Primary Cell Culture

After explantation, tumours were mechanically disrupted and washed in RPMI 1640/Hepes medium (Life Technologies Ltd., Paisley, U.K.), supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin (Sigma), and incubated for 30 min. at 37°C in PBS containing 0.5 mg/ml Collagenase H (Boehringer Mannheim GmbH, Mannheim, Germany); digestion was stopped by washing with medium containing 10% foetal calf serum (FCS). Cells were centrifuged at 700g, resuspended in medium supplemented with antibiotics, 20% FCS, 1 mM sodium pyruvate, 2 mg/ml glutamine, 100 mg/ml heparin (Sigma) and 30 µg/ml endothelial cell growth supplement (ECGS, Sigma), and plated in dishes previously coated with 1% gelatin (Sigma) in complete medium. Cells were maintained in culture until the monolayer was achieved, then treated with trypsin and replated in complete medium without heparin and ECGS, supplemented with 10% of their own conditioned medium.

2.6 Lymphangioma cells trasplantation

Cells were harvested by typsinization, washed in PBS and diluted to 1x10⁶ cells per ml in PBS. Cells were injected intraperitoneally in syngenic mice (Balb/c, Swiss); three to eight weeks after injection the mice were sacrificed and the peritoneal area evaluated.

2.7 Cell lines and transfection

The murine myeloma SP20 (ATCC, Rockville, USA) was maintained in RPMI 1640 supplemented with 10% FCS, 2 mg/ml glutamine, penicillin (100

U/ml), streptomycin (100 µg/ml) and 1 mM sodium pyruvate. The NIH3T3 murine fibroblast (ATCC, Rockville, USA) and the murine monocyte/macrophage J774A.1 cell lines were maintained in DMEM with 10% FCS, 2 mg/ml glutamine and the same antibiotics listed above. The Chinese Hamster Ovarian (CHO) cell line (ATCC, Rockville, USA) was growth in α-MEM medium supplemented with 10% serum and the same components described for DMEM.

CHO transfection was performed by electroporation. 2×10^6 cells were washed, resuspended in 500 µl cold PBS, mixed with BglII linearized plasmidic DNA (10 µg in 20 µl H_2O) and electroshocked with one pulse using the Bio-Rad Gene Pulser at 250 volts and 960 µF. After 5 min. on ice, the cells were washed twice, suspended in 30 ml medium and seeded into three Petri dishes (100 mm diameter) for selection and cloning by G-418 (Geneticin, Life Technologies, Inc., Gaithersburg, MD) at a concentration of 500 µg/ml in culture medium. Transfected cells were cultured for three weeks for colony formation. Colonies were separately harvested and positive clones were identified by western immunoblotting.

2.8 β-Galactosidase staining

For detection of β-gal activity in mice tissues, cryocut tissue slides were fixed in 2% gluteraldehyde solution for 5 min. at 4°C, washed with PBS three times for 10 min. and later incubated in stain solution (8.4 mM KCl, 1mM $MgCl_2$, 3mM $K_4FE(CN)_6$, 3mM $K_3Fe(CN)_6$, 3% 5-bromo.4-clore-3-indolyl β-D-galactopyranosyde (X-Gal) overnight at 37°C.

This experiment was performed in Haartman Institute, University of Helsinki, Finland, by Lotta Iussila. The heterozygous knock-out Flt-4$^{+/-}$ mouse, which contain a β-galactosidase expression cassette under the control of the Flt-4 gene promoter used in the assay, is a animal model kindly provided and developed by Kari Alitalo in collaboration with Daniel J. Dumont (74).

2.9 RNA extraction and RT/ PCR analysis

Umbilical cords were obtained from pregnant mice at 18 days postcoitum. Aortas were collected from adult animals and the adventitia tunica was surgically separated from the media and intima. Total RNA was extracted from the tumour, from mouse normal tissues and mouse cell lines with a standard method (50).

For RT/PCR, a single reverse transcription reaction was performed on each RNA sample using 1 μg of total RNA and the Gene Amp RNA-PCR kit (Perkin Elmer Cetus, Norwalk, CT, U.S.A.) with oligo-dT as primer. Each reverse transcription reaction was divided into aliquots and PCR amplification was performed with sets of primers specific for defined regions of the different transcripts as reported in Table II).

In order to perform RT/PCR analysis of chemokines expression, total RNA was isolated from different tumours using the acid guanidinium thiocyanate procedure. Aliquots from each preparation were mixed and two micrograms of the total RNA mixture were reverse transcribed following the above protocol. PCR amplification was performed with sets of primers specific for the different chemokine coding regions as reported in table II.

For each sample thirty five cycles of PCR were done under the following condition: 1 min. denaturation at 95°C, 1 min. annealing at 58°C and 1min. extension at 72°C. The identities of all PCR products were confirmed by Southern blot analysis using as probes [^{32}P]-end labelled oligonucleotides specific and internal to the amplified fragments (TAB. II). Oligonucleotides were purchased from Primm s.r.l., Milano, Italy.

2.10 Southern Blot analysis

Five microliters of each PCR fragments were loaded and separated in 1-1.5% agarose gel and overnight transferred to nylon membranes (Hybond-N, Amersham, UK) by Southern methods. After transfer, DNA was U.V. cross-linked to membrane. Filters were prehybridized for 1h at 42°C with SET 2X/ 0.25% v/v skimmed milk and hybridized in the same solution for 4h at 42°C with the specific internal oligonucleotides [γ-^{32}P] ATP labelled. The reaction for 50 ng of oligonucleotides was carried out in a total volume of 50µl in presence of 10 units of T4 Polynucleotide Kinase (BioLab, U.K.) and 20 µCi [γ-^{32}P] ATP (Amersham U.K., Ltd.). Samples were incubated at 37°C for 30' and radiolabelled fraction was purified from unincorporated nucleotides by in Nick-Column (Pharmacia, LKB) filtration. After hybridization filters were washed twice in SET 2X/ 0.1% SDS, twice in SET 1X/0.1 %SDS and once in 0.1X SET/0.1% SDS for 15 min. between 42°C and 48°C and autoradiographed o.n. at -70°C using X-O-Mat "S" (Kodak)

CDNA		Primers	Probes
VEGF 586bp	F	5'-TCCGAAACCATGAACTTTC-3'	5'-CTCCAACCATGCCAGTGGT-3'
	R	5'-TGGCTCACCGCCTTGGCT-3'	
Flk-1 654bp	F	5'-GACAAGACAGCGACTTGC-3'	5'-ACCTTGGAGCATCTCATCTG-3'
	R	5'-AATTGTGTATACTCTGTCA-3'	
Tie-1 430bp	F	5'-GTGCTGGTCGGAGAGAACCTGGCCT-3'	5'-ACTACAGCGTTTATAC-3'
	R	5'-AGGTGAAGTTCTCAAACAGCGACAT-3'	
Tie-2 427bp	F	5'-AGTTGGTGAAAAACTACATAG-3'	5'-AGTGATGTCGGTCCTAT-3'
	R	5'-GGTAAACTTCTCATACAGTGTGGT-3'	
Flt-4 788bp	F	5'-TTGGCATCAATAAAGGCAG-3'	5'-CAGCGAGCACCGTGCCCT-3'
	R	5'-CTGCTGGGTGTACACCTTA-3'	
VWF 360bp	F	5'-TGGTCCGCTATGTCCAAGGT-3'	5'-AGTCAGCTACCTCTGTGACCT-3'
	R	5'-TCTTATTGAAGTTGGCTTCA-3'	
GAPDH 570bp	F	5'-ACATGTTCCAGTATGACTCT-3'	5'-GAGTATGTCGTGGAGTCT-3'
	R	5'-TGCCTTCCGGTACGGTCAC-3'	
RANTES 293bp	F	5'- GGTACCATGAAGATCTCTGCA-3'	5'-AGGAGTATTTCTACACCAG-3'
	R	5'-AAACCCTCTATCCTAGCTCAT-3'	
JE 464bp	F	5'-ACCACCATGCAGGTCCCTGT-3'	5'-AACCTGGATCGGAACCAAAT-3'
	R	5'-GAGTCACACTAGTTCACTGT-3'	
IP-10 331bp	F	5'-CCCCATCAGCACCATGAAC-3'	5'-ATAGGGAAGCTTGAAATCAT-3'
	R	5'-GCTTCACTCCAGTTAAGGA-3'	
KC 312bp	F	5'-GTACCATGATCCCAGCCAC-3'	5'-AACATCCAGAGCTTGAAGGT-3'
	R	5'-GTCTTCTTTCTCCGTTACTTG-3'	
MIP-2 303bp	F	5'-ACTTCAGCCTAGCGCCATGG	5'-AACTGCGCTGTCAATGCCTG-3'
	R	5'-AGGTCAGTTAGCCTTGCCTT-3'	
C10 362bp	F	5'-CAGGAGGATGAGAAACTCCA-3'	5'-TATGCCACACAGATCCCATG-3'
	R	5'-CTTCTCAAGCAATGACCTTG-3'	
Lpnt 360bp	F	5'-CTCAGCCATGAGACTTCTC-3'	5'-AAAGCAGCGATCAAGACTGT-3'
	R	5'-TGGAGGCTGTTACCCAGTCA-3'	
BLC 314bp	F	5'-AGAATGAGGCTCAGCACAGC-3'	5'-ATAGATCGGATTCAAGTTACG-3'
	R	5'-TAGTGGCTTCAGGCAGCT-3'	
6Ckine 409bp	F	5'-ATGGCTCAGATGATGACTCT-3'	5'-AGCCTGGTCCTGGCTCTCTGC-3'
	R	5'-TACTGGGCTATCCTCTTGA-3'	
MIG 398bp	F	5'-TGCCATGAAGTCCGCTGTTC-3'	5'-ATAAGGAATGCACGATGCTC-3'
	R	5'-AAAGTAATGGTCTCTTATGTAG-3'	
EXODUS 412bp	F	5'-AACTCAACCACAATCATGGC-3'	5'-GACCAGGCTCTCTGCATC-3'
	R	5'-GCTATCCTCTTGAGGGCTGT-3'	
MIP1-α 294bp	F	5'-AACATCATGAAGGTCTCCAC-3'	5'-TCATCGTTGACTATTTTGAA-3'
	R	5'-CCAAGACTCTCAGGCATTCA-3'	
MIP1-β 293bp	F	5'-ACACCATGAAGCTCTGCGT-3'	5'-AGACCAGCAGTCTTTGCTCC-3'
	R	5'-CGCTGGAGCTGCTCAGTTC-3'	
MIP1-γ 390bp	F	5'-TAAGAAGATGAAGCCTTTTCA-3'	5'-ACTCTTCAGATTGCTGCCTG-3'
	R	5'-TCTCTAAAGCAAATGTTATTGT-3'	

Table II: Nucleotide sequences of primers used for RT/PCR analysis and Southern Blot analysis. In the first column are indicated the transcripts of interest and the expected size of amplified fragments. In second column are reported forward (F) and reverse (R) primers for PCR

PCR amplification. In the third column are indicated the oligonucleotides used as probes in Southern blot analysis of the transcripts.

2.11 Cloning and Sequencing

PCR fragments obtained by amplification of lymphangioma cDNA were cloned in pGEM -T Easy vector (Promega, Madison; WI, USA) following the manufacturer's recommendations, and used to transform *E. coli* strain DH5-α. At least three positive clones from each transformation were sequenced using the T7 Sequencing kit (Pharmacia LKB).

The C10 amplified fragment was purified from 1% agarose gels by electroelution. The recovered DNA fragment was blunted and phosphorylated by treatment with Klenow and Kinase enzyme (BioLab, UK) and ligated in the SmaI site of PUC18 (Pharmacia LKB, Uppsala, Sweden). 1μg of Puc 18/C10 construct was EcoRI/HindIII digested (BioLab, UK), the 374 nucleotides fragment purified and subcloned into the EcoRI/HindIII cloning sites of the pcDNA3 vector (Invitrogen, CA, USA). Maxi preparation of the plasmid pcDNA3/C10 was obtained with the Quiagen plasmid kit (Quiagen, Germany) and the concentration determined by spectrophotometry.

2.12 Northern Blot analysis

Total lymphangioma RNA (20 μg/sample) was separated by electrophoresis in denaturing 1% agarose gel containing 6% formaldehyde, transferred to nylon membranes (Hybond-N, Amersham, UK) by overnight capillary blotting in 20 X SSC and fixed with UV light. The filters were

hybridized with different probes generated by convenient restriction enzyme digestion of the PCR amplified fragments cloned in pGEM-T Easy Vector (Promega, Madison, WI, USA). The size of probes are reported in table II. Probes were labelled with [α-^{32}P] dCTP (Amersham, UK) using the oligolabelling kit (Pharmacia, LBK).

Hybridizations were carried out overnight at 50°C in 5X SSPE/0.5% SDS/5X Denhardt's/ 50% formamide/ 100 mg/ml salmon sperm DNA. Filters were washed in 2XSSC/ 0.1% SDS at 50°C, 60°C and 70°C for 30' and autoradiographed overnight at -70°C using X-O-Mat "S" (Kodak).

Total RNA of mouse skeletal muscle cells was included as control.

2.13 *In situ* Hybridization

In situ hybridizations were performed basically as described by Komminoth et al. (154) with modifications. 5 μm thick, formalin fixed, paraffin-embedded tissue sections were placed on Superfrost Plus-treated slides (Fisher Scientific). Tissue sections were deparaffinized, hydrated and permeabilized with 0.5-0.2 μg/ml proteinase K for 30 min. at 37°C, and prehybridized in hybridization buffer containing 50% formamide, 50 mM HEPES, 5X Denhardt's solution, 4X SSC, 250 μg/ml denatured herring sperm DNA and 5 mM EDTA, pH 8.0 for 2h at 42°C. Hybridization was performed overnight at 56°C in a humidified chamber (5-10 ng of digoxigenin-labeled RNA probe in 200 μl of hybridization buffer/slide). As a negative control, sections were treated with RNAse A prior to prehybridization, and a second negative control was performed using the corresponding sense RNA as a probe. All the templates were obtained by amplification of lymphangioma

cDNAs with the oligonucleotides above described (table II), inserted into the pGEM-T plasmid vector (Promega) linearized and transcribed with SP6 RNA polymerase or T7 RNA polymerase (Promega) for the synthesis of the antisense and sense RNA probes, respectively (TAB. III). RNA probes were labelled with DIG-UTP using the Dig RNA labelling mix (Boehringer Mannheim).

After hybridization, unbound probe was digested with RNAse A (20 µg/ml) for 30 min. at 37°C. Post-hybridization washes were all performed at 37°C and consisted of two washes in 2X SSC, two in 0.2X SSC, and one in 0.1X SSC (each for 15 min.). The hybridization signal was detected according to the instructions of the Dig Nucleic Acid Detection kit (Boehringer Mannheim), using Fast Red as a substrate of alkaline phosphatase. The slides were counter-stained with Harris solution (Sigma), and mounted with glycerol/phosphate-buffered saline (50% v/v).

TEMPLATE	Res. Enzyme/S	Res.Enzyme/A	Probe Size
ICAM-1	Sal I		431bp
ICAM-1		Nco I	431bp
FLT-4	Sma I		468bp
FLT-4		Sma I	217bp
MIG-1	Sal I		398bp
MIG-1		Nco I	398bp
JE	Spe I		504bp
JE		Hpa I	436bp
Lptn	Spe I		462bp
Lptn		Nco I	441bp
C10	Eco R I		374bp
C10		Hind III	374bp

TableIII: PCR fragments cloned in PGEM-T easy vector are linearized in order to synthetize sense and antisense RNA probes.

2.14 Western Immunoblotting

Extracts of lymphangioma or cultured cells were prepared by homogenization in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA) with protease inhibitors PMSF (1 mM), aprotinin (10μg/ml), and leupeptin (10 μg/ml) (all from Sigma Chemical Co.). Immunoprecipitations were performed by incubating the lysates with rabbit-anti mouse Flt-4 or rabbit anti-mouse Flk-1 antibodies, followed by addition of 50 μl of Protein-A Sepharose (Pharmacia Biotech, Uppsala, Sweden). Precipitates were washed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris-Hcl pH 7.5), eluted with 0.1 M Glycine HCl pH 2.5 and neutralized with 10 μl 1M Tris HCl pH 8.0. Aliquots were run on an reducing 7.5% SDS-PAGE, blotted onto Hybond ECL nitrocellulose membrane (Amersham International, Little Chalfont, U.K.) and reacted with the same antibodies, followed by horseradish peroxidase-coupled anti-rabbit Ig antibody (DAKO). Treatments with recombinant N-glycosilase F (PNGase f) were done according to the protocols provided by the manufacturer (New England BioLabs Inc., Beverly, MA, U.S.A.). Detection was performed using the ECL kit (Amersham International) and membranes were exposed to a Kodak Biomax film for 1min.

Aliquots of the same immunoprecipitated samples were incubated with an irrelevant antibody (goat anti-human IgE(ε), Kirkegaard &Perry Laboratories, Gaithersburg, MA, U.S.A.), used as negative control.

Supernatants of different pcDNA3/C10 transfected CHO clones were collected and the same inhibitors listed above were added. The supernatants were cleared by centrifugation at 10000g for 15 min. at 4°C. Subsequently,

50μl of each supernatant were subjected to 12% SDS-PAGE and Western blotted. The membrane was incubated with a 1: 100 dilution of goat anti-mouse C10 (Sigma), followed by horseradish peroxidase-coupled anti-goat Ig antibody (DAKO). The bound antibodies were detected by ECL.

2.15 Separation of Murine Haemopoietic Subpopulation

For isolation peripheral blood neutrophils and lymphocytes, blood was collected from the retro-orbital plexus of CD1 mice (JacKson Laboratories, Milano, Italy) into pipettes containing EDTA anticoagulant. Blood was then incubated with an equal volume of 3% dextran in 0.15 M NaCl. After erythrocytes sedimentation, the leukocyte-rich plasma was collected and washed twice with PBS/0.5% BSA (Sigma). Cells were resuspended in 2 ml PBS/0.5% BSA and layered over a five-step discontinuous Percoll 9% NaCl gradient (Pharmacia, LKB), as previously described (276) . After centrifugation at 500g at 20°C for 20min., the cells were banded at the surfaces of the gradient densities, and fractions 3 and 5 were collected separately. This procedure resulted in a preparation greater than 95% of neutrophils from fractions 3 and 5 and a preparation greater than 95% of lymphocytes from fraction 1. Cell viability was >98% as assessed by trypan blue dye exclusion. Harvested cell were washed twice and suspended in PBS/0.5% BSA, at 2×10^6 cells/ml.

For isolation of resident peritoneal macrophages, mice were killed by cervical dislocation and the peritoneal cavities were washed out with 7-10 ml PBS. The harvested cells were then washed twice and resuspended in 2ml

PBS/0.5% BSA. Cell suspension was then layered over 4ml Percoll suspension of 1.0830±0.0003 density and osmolitic value of 310 2m OSM. After centrifugation at 400g for 20min. at 20°C, the cell ring formed at the PBS/Pecoll interface was collected, washed twice and resuspended in PBS containing 1% BSA. The resulting cell suspension contained >95% macrophages as assessed by morphology and peroxidase-staining, and cell viability was >98% as determined by trypan blue exclusion.

Mouse peritoneal neutrophils were isolated from CD1 8-12 weeks old mice after intraperitoneal injection of 3% thioglycolate broth (BBL Microbiology System, ockeysville, MD). 4h later, peritoneal cells were harvested in 7-10 ml PBS (68). Separation of neutrophils from the peritoneal exudate was performed by using a Percoll gradient. Peritoneal cells suspended in 2ml PBS/0.5% BSA were layered over 4ml Percoll (with density and osmotic value above reported). The gradient was centrifuged for 20 min. at 400g at 4°C. The resulting cell pellet was washed twice with PBS and neutrophils were tested for purity by examination of eosin haematoxylin stained cytospin preparations. All neutrophil preparations were >95% pure.

2.16 Separation of human neutrophilic polymorphonuclear cells

Human PMN were isolated from acid-dextrose (ACD)-anticoagulated blood by Ficoll-Hypaque (Nycomed Pharma AS, Oslo, N), gradient centrifugation, 3% dextran (Pharmacia) sedimentation and hypotonic saline lysis of contaminating erytrocytes (31). The entire procedure was conducted at 4°C. Purified PMN were suspended in PBS containing 1% BSA.

2.17 Chemotactic Assay

The *in vitro* migration of leukocytes was assessed in a 24-well transwell chamber with a 5 or 1.2 μm pore polycarbonate Transwell culture insert (Costar, Cambridge, MA) as described previously (27). Briefly, leukocytes were suspended in RPMI 1640 medium containing 10% fetal calf serum, at $2.5x10^6$ cells/ml. The lower wells were filled with 600 μl of lymphatic endothelial cells conditioned medium (LCM) or control medium containing 1% Zymosan activated serum (ZAS), 25 ng/ml MIP-1α (Genzyme, Ma, USA) or C10 transfected CHO cells supernatant. The upper wells were filled with 100 μl of cell suspension. Assay were carried out at 37°C for 30 min. for neutrophils, 1h for macrophages and 3h for lymphocytes. Cell migrated into the lower chamber were then harvested and counted in a ZBI Coulter Counter (Coulter Electronics LDT, Luton, UK). The results were expressed by mean (+/-) number of migrating cells in five or more random high-power fields tested in triplicate.
Significance of the value was determinate by paired T-Test (*p<0.0001).

2.18 ZAS Preparation

5 mg of lyophilised Zimosan (Sigma), were resuspended in 1 ml PBS and boiled 5 min. After centrifugation, 2000 rpm 10 min., the pellet was resuspended in 125μl PBS and added to fresh serum with a proportion of 1:4. This solution was incubated 30 min. at 37°C in order to activate the complement present in the serum and then centrifuged 12000 rpm 5 min..

The zymosan activated serum (ZAS) was recovered and added at 1% into the medium.

Chemotactic assays were performed in collaboration with Dr. Aldo Dobrina and Dr. Elena Vecile of the Dipartimento di Fisiologia e Patologia, Università di Trieste.

Chapter 3

RESULTS

LYMPHANGIOMA CHARACTERIZATION

3.1 Induction and histopathology of intraperitoneal tumours

The intraperitoneal injections of incomplete Freund's adjuvant into mice induced the development of multicentric and clearly delineated white solid masses, with the histological appearance of lymphangiomas. These lesions, varying in size from 5 to 50 mm^2 were localized in the peritoneal cavity along the thoracic duct, on both abdominal surfaces of the diaphragm and on the upper surface of both lobes of the liver (Fig. 12a). In some cases, they were also observed on the surface of the spleen.

We have tested more than 100 Balb/c mice and found that the lesion was induced in all (100%) injected animals. Smaller masses were evident even when the animals were injected with a single dose of the adjuvant. Although the lesion size was usually slightly larger in female mice, we did not observe any other difference related to sex or age. Other strains of mice such as AS/N, C57 black, and Swiss were also treated in order to exclude the possibility that lesion formation was related to the genetic background. In all three strains the same efficiency of response (100%, 15 animals each) was

observed even though the sizes of the lesions were usually smaller than in Balb/c mice. Furthermore, we observed that pristane, a mineral oil widely used to establish ascitic growth of myeloma or hybridoma cells (161) was also effective in inducing the development of peritoneal masses.

The white colour of the masses suggested that the lesions were caused by developing lipomas; in order to verify this hypothesis Sudan III staining was performed on cryocut sections. Actually, as indication of lipids presence, the staining was strongly positive, but the microscopical analysis revealed that the lesions were not formed by adipocytes. The lipidic material was not stored in adipocytes liposomas, but in lumina surrounded by a variable number of cells, as indicated by the nuclei pointed out (Fig. 11).

Histopathological analysis of the lesions after haematoxylin/eosin staining revealed the presence of endothelial cells surrounding empty lumina. Various levels of vascular development were present. Observed cells showed intracytoplasmic vesicles of different sizes and fused to neighbouring cells forming vessel-like lumens (Fig. 12c, d). The lesions were not infiltrated with either erythrocytes, lymphocytes or platelets. However, recruitment of granulocytes was frequently observed in the region of the liver close to the lesion (Fig.12b). No evidence of direct infiltration of the organ parenchyma was observed. On the surface of the liver, tumour growth was restricted to a region under the Glisson's capsule, with no infiltration into the hepatic parenchyme (Fig.12b-d) or metastasis. When the mice were sacrificed six or eight months later, in absence of stimuli, no progression, but still persistence of the lesion was observed. Only in a single case the described lesion was accompanying a neoplastic formation detected in an axillo lymph node.

a

b

Figure 11. Sudan III staining of lymphangioma section.
Different magnification (a-b) of a frozen section of lymphangioma lesion
stained with Sudan III, a staining specific for the identification of lipids. Cell
nuclei are stained in purple, cytoplasma in grey. Red spots identify different
size extracellular areas of lipids accumulation, corresponding to the lumina
revealed in the lesion as seen in Fig. 12.
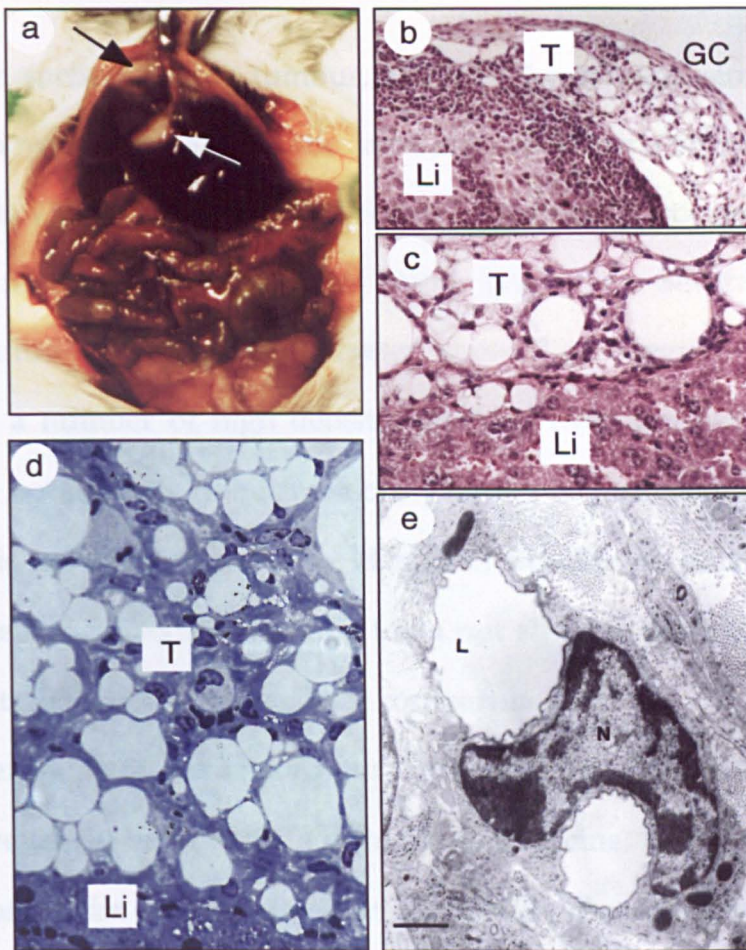
Figure 12. Anatomical localization, histological analysis and electron microscopy of lymphangioma.

(a) Peritoneal cavity of a Balb/c mouse showing the white plaques of the lymphangioma tumour growing on the peritoneal surface of the diaphragm (black arrow) and on the surface of the liver (white arrow). (b-d) Histological section of the liver lesion, stained with hematoxylin and eosin (b, c) or toluidine blue (d). It is apparent that the tumour grows under the Glisson's capsule (GC) without infiltrating the hepatic tissue. On the boundary between the tumour (T) and liver (Li), a collection of leukocytes is present without evidence of tissue or cell necrosis (b). At higher magnifications (c-d) vacuolated cells and fused cells at different stages of lumen formation can be seen. Vesicles of different sizes, in some cases confluent (d) are present. e, Electron microscopic picture of a single lymphangioma cell containing two lumens with irregular borders. Cross and longitudinal sections corresponding to intermediate filaments are also evident. N, nucleus; L, lumen (Bar, 1nm).

Ultrathin sections of the tumour, analysed by transmission electron microscopy, showed the characteristic formation of lumens surrounded by endothelial cells. Often, a single endothelial cell was found participating in the formation of more than one luminal cavity (Fig. 12e). Several vesicles, especially the ones with a smaller diameter, showed an irregular outline. In the cytoplasm a number of high density granules were present, while no Weibel-Palade bodies, the characteristic organelles where von Willebrand Factor (vWF) is stored (296) could be identified. Bundles of collagen fibrils, revealed by their characteristic banding (data not shown), were observed in different orientations in the extracellular compartment.

Considering that: "The word tumour means literally an abnormal swelling. However, in the language of modern medicine, the word assume the meaning of *a lesion resulting from the autonomous abnormal growth of cells which persist after the initiating stimulus has been removed.*" (287), it will be correct think of the described lesions as a tumour of (lymphatic) endothelial cells.

3.2 In vitro propagation of tumour derived cells

The lymphangioma tumours could be easily explanted and separated from the surrounding tissues. The endothelial cells recovered after treatment with collagenase grew *in vitro* (Fig.13) in an anchorage dependent manner: the dishes had to be coated with gelatin for the successful establishment of the primary cultures. When cultivated in the presence of bovine vascular endothelial growth factor or a crude bovine pituitary extract, the cells
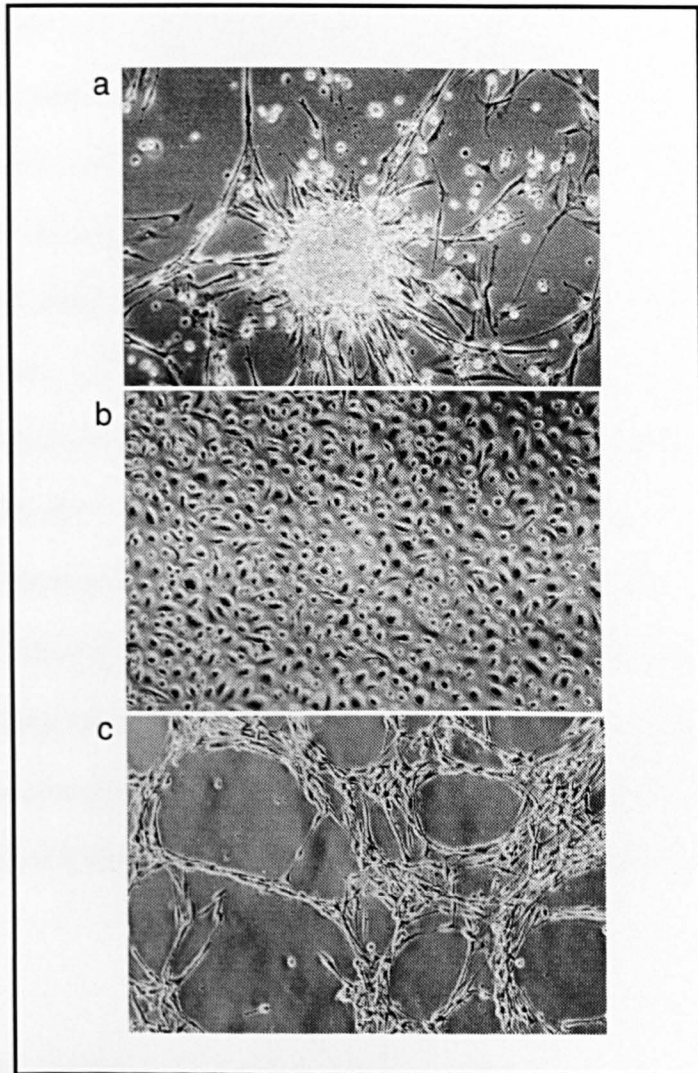
Figure 13. Primary culture of lymphangioma derived cells.

Phase contrast images of cells after 48hrs of plating (a), at confluence after
approximately 10 days of colture and after three passages (c), where spontaneous
formation of elongated structures can be seen. The culture is performed in dishes
previously coated whith 1% gelatin in complete medium. Initially (a-b) cells are
growing in complete medium supplemented with 30mg/ml of endothelial cell
growth supplement (ECGS) or in presence of pituitary extract (1:200 in medium).
Replated cells were cultured in complete medium supplemented with 10% of their
own conditioned medium. Lymphatic endothelial cell conditioned medium is
indicated as LCM.

proliferated, albeit at a low rate with a doubling time of about 48-60 hours, reaching confluence in eight to ten days. At this stage, the lymphangioma cells showed a classical cobblestone morphology (Fig. 13a) and further cell proliferation was contact inhibited. After the third/fourth passage, cellular growth slowed down and the capacity to reach a high cell density was lost. An increased number of cytoplasmic vacuoles was also evident in these cells. After a period of two to three months, the cells entered a quiescence/senescence state and died. At this stage, cellular morphology changed drastically with cells becoming multinucleated with a large cytoplasm. Nevertheless, during the initial period of culture (usually after 5 to 20 days) spontaneous formation of characteristic vessel-like structures was observed, starting from the lengthwise pairing of a few cells (Fig. 13b, c). The capacity of primary cultures of lymphatic endothelial cells to form vessel-like structures has been shown for bovine cells from the thoracic duct (210, 229).

3.3 Trasplantation of lymphangioma cells

In order to evaluate the growth potentiality of the lymphatic endothelial cells in syngeneic, untreated host, transplantation experiments were done. Balb/c and Swiss mice were sacrificed and analysed macroscopically after three-eight weeks from the intraperitoneal injection of lymphangioma cells. Any animal didn't shown evidence of tumour formation neither in the injection site, nor on organs surfaces in the peritoneal area.

3.4 Expression of endothelial markers

To further characterize the tumours we examined the expression of endothelial cell markers such as the VEGF-receptor Flk-1 and other receptor tyrosine kinases Tie-1, Tie-2 and Flt-4 (155, 238, 72, 88) and the intracellular vWF. In addition, the expression of the vascular endothelial growth factor (VEGF) (147) (171) was investigated in the lesions. The analysis was by means of specific RT/PCR of defined regions of the corresponding mRNAs, followed by hybridization with probes specific for the amplified segments. As a control of normal mouse endothelium, we used mRNA extracted from the umbilical cord and the vascular endothelium of the aorta, as well as from the adventitia of the aorta which is rich in lymphatic vessels and does not contain blood vessels. Other controls included mRNA from adult mouse liver, from NIH 3T3 cells and from the myeloma Sp2/0. A single reverse transcription reaction was divided in equal aliquots that were subsequently amplified by PCR with the corresponding specific oligonucleotides. Negative controls were included for all amplifications.

As shown in Figure 14a, the VEGF mRNA was expressed by the lymphangioma, aorta and NIH-3T3 and Sp2/0 cells, but little or no VEGF mRNA was detected in the adventitia or the umbilical cord. The amplified bands corresponded to two previously described alternatively spliced VEGF isoforms differing by 72 nt, corresponding to the exon 6 (285). The identities of these bands were confirmed by sequencing (not shown). The longer form, which encodes a mature protein of 188 aa was expressed more abundantly than the 164 aa form in the lymphangioma. The major mitogenic receptor for VEGF, Flk-1, was expressed in lymphangioma, umbilical cord and aorta,
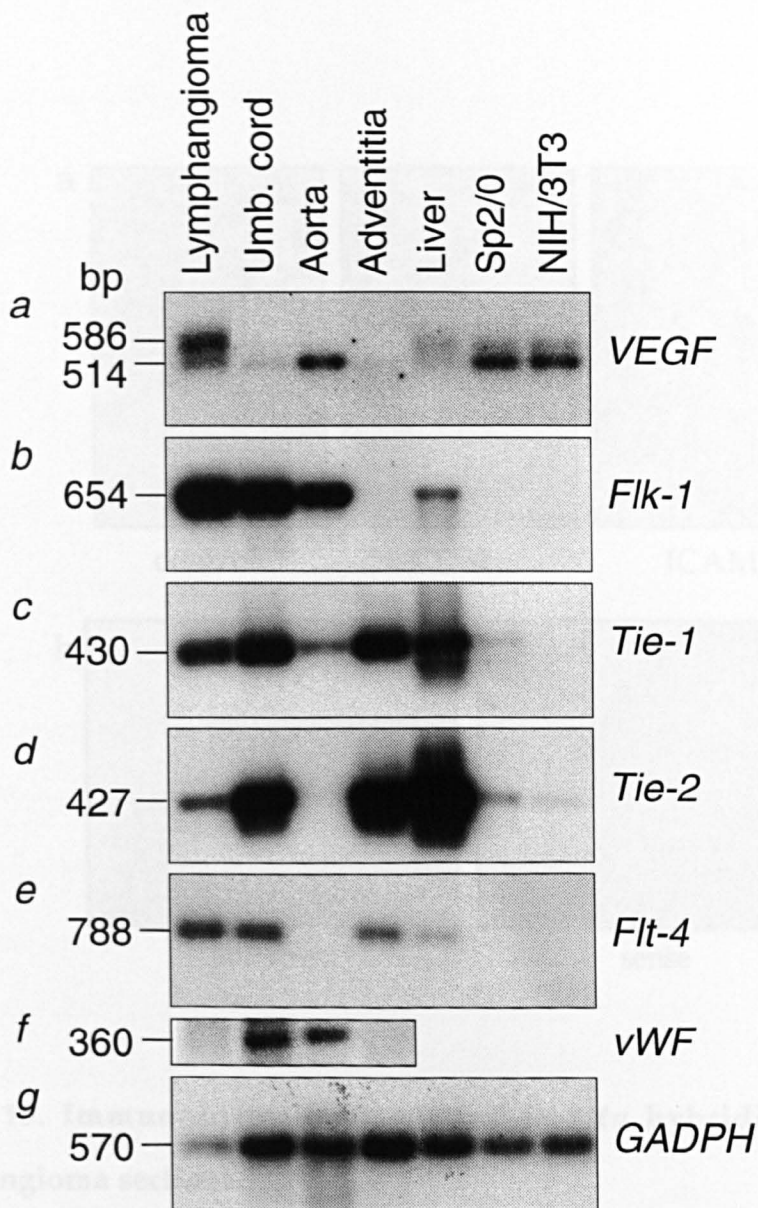
Figure 14. RT/PCR analysis of Expression of Endothelial Markers. Each panel shows autoradiograms of hybridizations with probes specific for the amplified segments of mRNA, as described in Matherials and Methods (sections 2.8). Sizes, in base pairs (bp), of the different amplified fragments are indicated on the left. For each RNA sample, a single reverse transcription reaction was carried out and equal aliquots were used for the different PCR amplifications. a-g panels correspond respectively to: a) Vascular Endothelium growth Factor (VEGF), b) Fetal liver gene kinase-1 (FLK-1), c-d) Tunica interna endothelial cell kinase -1 (Tie-1) and -2 (Tie-2), e) fms-like tyrosine kinase gene-4 (Flt-4), f) von Willebrand Factor (vWF) and g)glyceraldehyde-3-phosphate-dehydrogenase (GAPDH).

control   CD31   ICAM2

antisense   sense
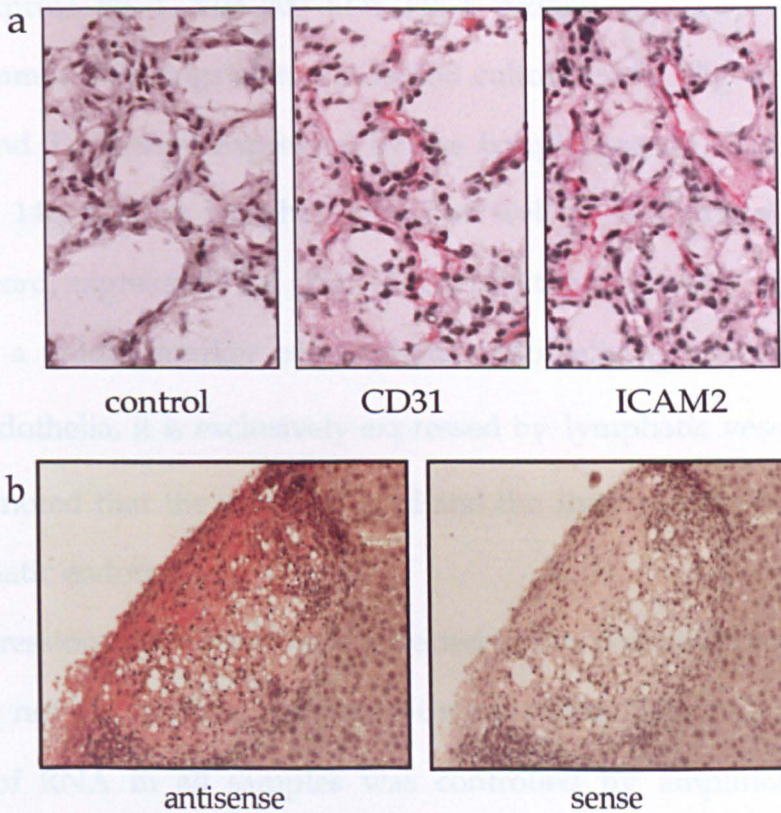
Figure 15. Immunohistochemistry and _in situ_ hybridization of lymphangioma sections.

Analysis of endothelial markers was performed on frozen sections of lymphangioma by immunohistochemistry (a). Slides were incubated with antibodies for PECAM/CD31 and ICAM2/CD102. As a second antibody a biotin-coniugated goat anti rat was used. The reaction was developed with Fast Red and slides counterstained with hematoxylin. The control section was stained with rabbit serum. (b) Paraffin embedded sections were hybridized with digoxigenin-labelled sense and antisense RNA probes for ICAM-1, as indicated. The hybridization signal was detected using Fast Red as a substrate of alkaline phosphatase and Harris solution was used for counter-staining.

but not in the adventitia (Fig. 14b). As expected, also the two control cell lines did not express Flk-1. The 200 kDa Flk-1 protein was also identified in Western immunoblottings of tumours and cultured cells (Fig. 16, lane 4 and 5). Tie-1 and Tie-2 were expressed by the lymphangioma, but not by the aorta (Fig. 14c, d). The lymphangioma, as well as the adventitia and the umbilical cord, expressed Flt-4 (Fig. 14e), while the aorta was negative. Flt-4 represents a unique marker of lymphatic endothelial cells since within the mature endothelia, it is exclusively expressed by lymphatic vessels (140). It should be noted that the umbilical cord and the liver contain both vascular and lymphatic endothelium.

Expression of vWF was also detected in the umbilical cord and in the aorta, but not in the lymphangioma or adventitia (Fig. 14f). Finally, the presence of RNA in all samples was controlled by amplification of the constitutively expressed glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA (Fig. 11g).

The expression of the endothelial cells markers PECAM/CD31, ICAM-1/CD54 and ICAM-2/CD102 was investigated by immunohistochemistry. PECAM/CD31 was positive with a heterogeneous staining (figure 15a), while ICAM-2/CD102 and ICAM-1/CD54 (not shown) were only weakly stained in the lymphangiomas. By *in situ* hybridization, ICAM-1 was clearly positive only in the region corresponding to the tumour (Fig.15b) and ICAM-2 was easily detected by RT/PCR (not shown) therefore the weakly staining is probably due to antibody limiting specificities.

3.5 Analysis of Flt-4 expression in the lymphangiomas

Expression of the Flt-4 lymphatic endothelial receptor for VEGF-C and VEGF-D was also investigated by Western blotting, by *in situ* hybridization and β-gal staining of tumours induced in heterozygous knock-out Flt-4$^{+/-}$ mice, which contain a β-galactosidase expression cassette under the control of the Flt-4 gene promoter.

Western blotting of anti-Flt-4 immunoprecipitates from either lymphangioma tumours or tumour derived *in vitro* primary cell cultures showed the 190, 170 and 120 kDa bands (Fig. 16), which are generated by differential glycosylation and proteolytic cleavage of the Flt-4 polypeptide backbone (223) In fact, after digestion with PNGase F, which removes N-linked glycosidic residues, the two larger Flt-4 of 190 and 170 kDa merged into a single band of about 130-140 kDa, while the major C-terminal proteolytic cleavage product of 120 kDa was reduced to 97 kDa (Fig. 16, lane 2). The N-terminal 75 kDa product was not detected in this assay because the polyclonal Abs used are directed to the C-terminus of Flt-4. The same three Flt-4 polypeptides were also identified in extracts from tumor derived cells cultured for one week (Fig. 16 lane 3). The negative control of the experiment is represented by samples from lymphangioma tumours or tumour derived primary cell culture treated as described with a goat anti-human IgE(ε).

Tumors induced in the heterozygous knock-out Flt-4$^{+/-}$ β-gal transgenic mice had a number of blue β-gal positive tumour cells (Fig. 17 a), although the staining was not as intense as in normal lymphatic vessels (data not shown). Flt-4 mRNA was also detected by *in situ* hybridization in a large number of cells within the tumour (Fig. 17 b, c).
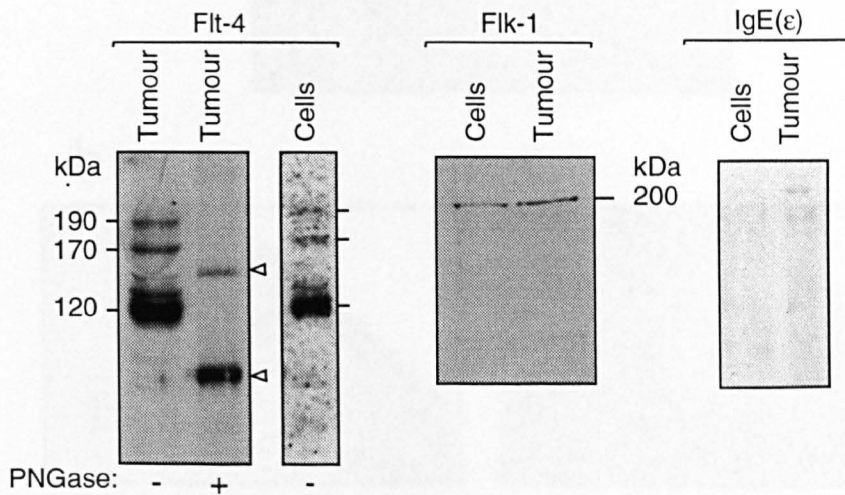
Figure 16. Western Immunoblot of Flt-4 and FlK-1.

Detection of Flt-4 (lanes 1-3) and Flk-1 (lanes 4-5) proteins by immunoprecipitation and western blotting from tumour or cultured cells, as indicated. PNGase F treatment of Flt-4 immunoprecipitates is shown on the right and relative masses are indicated in kDa. Negative controls are represented by samples immunoprecipitated and incubated with a goat anti-human IgE(ε).
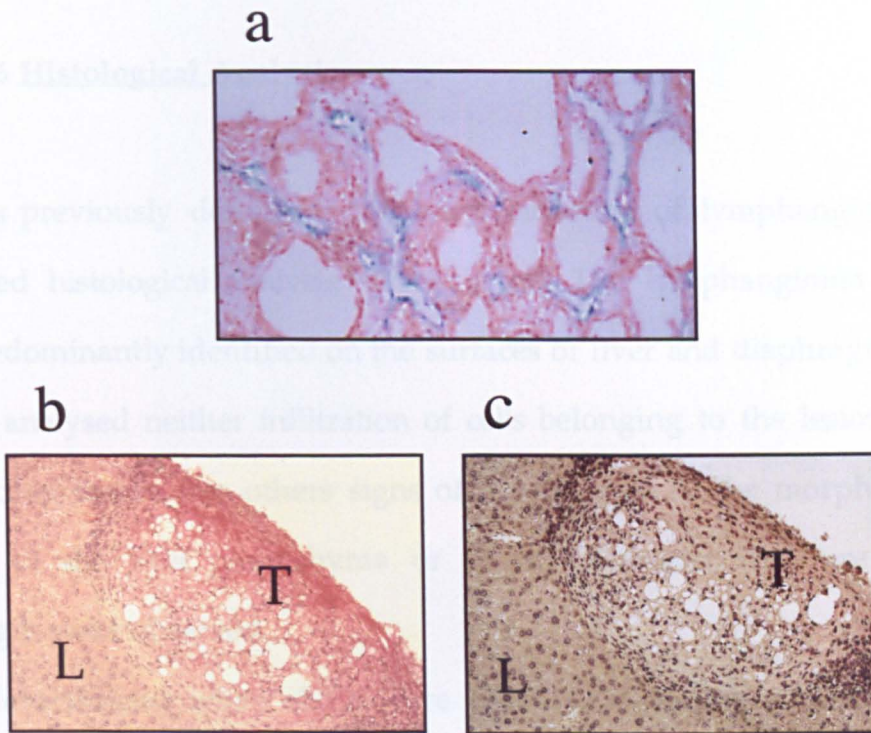
Figure 17. Flt-4 expression in lymphangioma.
Staining for β-galactosidase on frozen section of lymphangioma tumour induced in heterozygous knockout Flt-4 $^{+/-}$ β–gal mice (a). *In situ* hybridization of lymphangioma paraffin embedded sections with digoxigenin-labelled antisense and sense Flt-4 RNA probes is shown in (b) and (c) respectively. The hybridization signal was detected using Fast Red as a substrate of alkaline phosphatase and Harris solution was used for counter-staining. L, liver; T tumour.

CHEMOKINES EXPRESSION IN LYMPHANGIOMA

3.6 Histological Analysis

As previously described, following induction of lymphangioma we performed histological analysis of the lesion. The lymphangioma lesions were predominantly identified on the surfaces of liver and diaphragm. In all samples analysed neither infiltration of cells belonging to the lesion in the surrounding tissues nor others signs of modification of the morphological features of the liver parenchyma or of the muscular structure of the diaphragm were observed.

Nevertheless, when slides were eosin-haematoxylin stained, it was evident a remarkable presence of leukocytes in the area of the tissues neighbouring the lymphangioma. This observation was particularly evident in all histological samples in which liver fragments were analysed (Fig. 18).

Leukocytes seemed to be infiltrating the liver parenchyma closed to the developed lesions and often the extension of this infiltration seemed to be related to tumour size. In the liver region where the cells were accumulated no sign of necrosis was noticed. In addition, it was not observed invasion of leukocytes into the lymphangioma itself. The presence of leukocytes was also observed in areas relatively poor of blood venules and far from identifiable blood vessels. These observations suggested that the presence of leukocytes in the tissue surrounding the lymphangioma was probably related to a biological property of the lymphatic endothelial cells
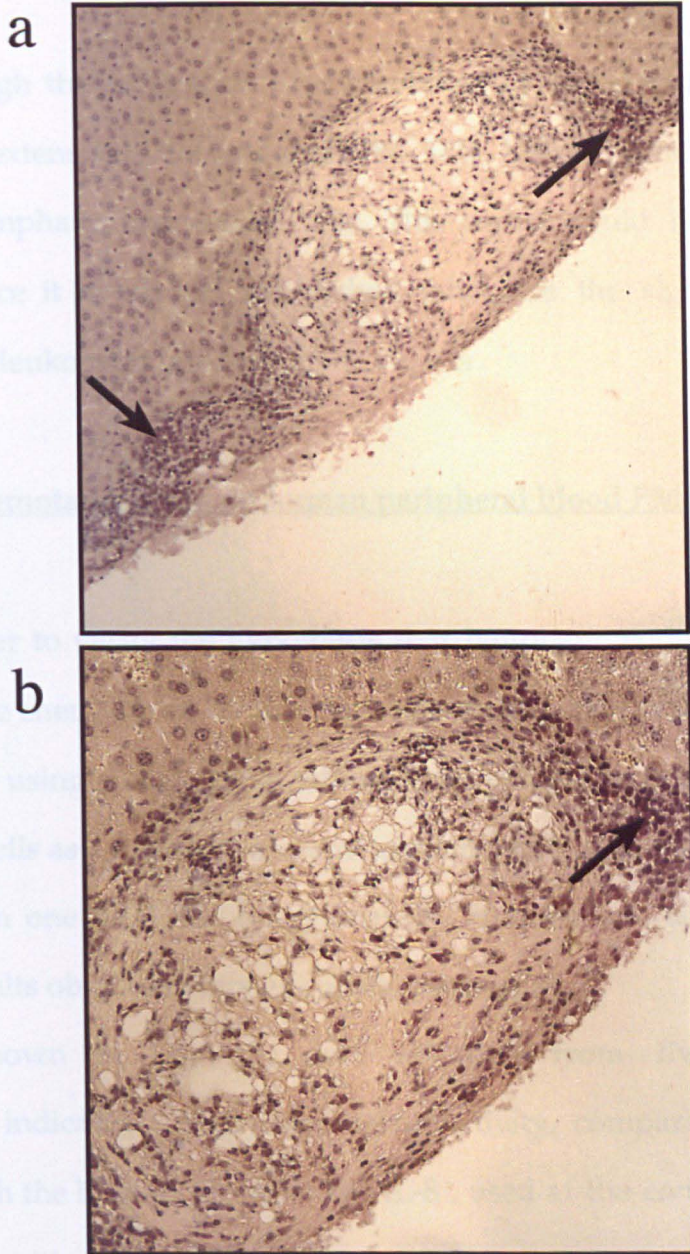
Figure 18. Identification of leukocyre recruitment by histological analysis.

Different magnification (a-b) of a histological section of lymphangioma lesion developed on liver surface. Arrows indicate a massive leukocyte recruitment in the area of the tissue neighbouring the lymphangioma.

Leukocytes were not infiltrating the surrounded lesion and no signs of necrosis is evident in the region where cells were accumulated.

represented by chemoattractant activity due to the production of chemokine.

Although the expression of chemokines by blood vessel endothelial cells is being extensively documented (184, 185), less information is available regarding lymphatic endothelial cells. This aspect could be of particular relevance since it is not yet established what are the signals that drive circulation of leukocytes into lymphatic vessels .

3.7 Chemotactic assay on human peripheral blood PMN

In order to verify the hypothesis that lymphatic endothelial cells are able to secrete chemokines, an in vitro chemotactic assay was performed on human PMN using culture supernatants from primary culture of lymphatic endothelial cells as a source of chemoattractant molecules. When conditioned medium from one week old lymphangioma cultures was tested on human cells, the results obtained were extremely promising.

As shown in Fig. 19, data recorded from five independent experiments indicated a high chemotactic activity, comparable to the one obtained with the human recombinant IL-8 , used at the concentration of 10 ng/ml as a positive control.

After 30 min. of incubation, more than 45% of human PBMC was able to transmigrate through the gelatinised membrane to the lower chamber, showing the same efficiency of IL-8. Chemotaxis activity by control with unconditioned media was reduced to 19-22%.

The comparison of the chemoatractant activity of the supernatant and the one of IL-8, that is thought to be in humans the primary regulatory

Human peripheral blood PMN
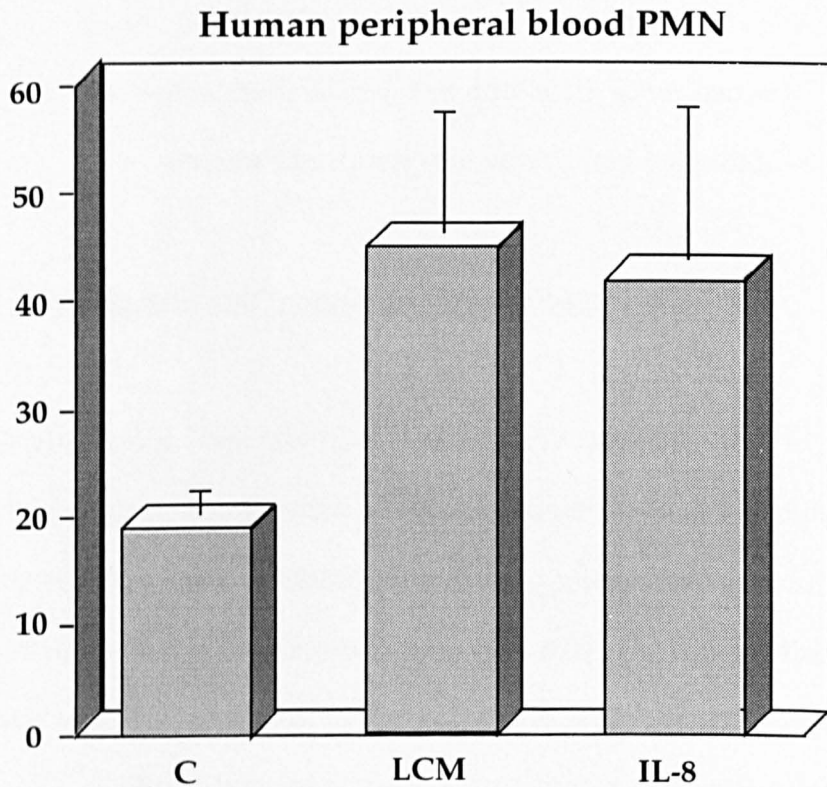
Figure 19. Chemotactic assay on human peripheral blood PMN.
Effect of lymphangioma derived endothelial cells conditioned medium LCM on chemotactic activity of normal human peripheral blood polymorphonuclear cells (PMN). The negative and positive controls are unconditioned medium (C) and IL-8 (10 ng/ml) respectively. Results are expressed as the percentage of input cells migrating into the lower chamber of a transwell filter (1.2 μm pore size) after 30 min. Each histogram represents the mean of five indipendent experiments and the significancy of the value between these conditions was determined by paired T-Test (p<0.0001).

molecule of acute inflammatory states, indicated that lymphangioma endothelial cells, as well as other better known models of activated endothelial cells (i.e. HUVECs) are able to secrete chemokine thus providing evidence that these molecule constitute at least in part, signals for trafficking of leukocytes through lymphatic vessels.

The kinetics of chemokine production by the lymphatic endothelial cells was next investigated using media conditioned for 48h at different moments from the establishment of the primary culture. The highest level of chemoattractant activity was recorded in cell culture derived from the 7th to the 10th day. After twelve days the chemoattractant activity on PBMC was significantly reduced. This observation indicates that *in vitro* chemokines production by lymphangioma derived endothelial cells is transient and probably related to the absence of appropriate stimuli.

3.8 Screening of chemokine expression in lymphangioma derived cells

In order to establish the profile of chemokines secreted by lymphatic endothelial cells, we investigated the expression of the different chemokines by means of specific reverse transcription/PCR of defined regions of the corresponding mRNAs.

For this assay total RNA was obtained from lymphatic cells after tumour explantation or from cultured cells.

Appropriate oligonucleotides were designed from mRNA sequences of mouse chemokines obtained from EMBL/GenBank/DDBJ database for each chemokines considered in our screening. Two primers were

synthesized in order to amplify the complete coding region and a third one, homologous to an internal sequence, for specific hybridization of the amplified fragments.

During the last year the progress in the chemokines field has been extremely rapid with a continuous growth of the number of human and mouse new chemokine sequences identified. We restricted our investigation to verify the expression of only the better known chemokines.

As shown in Fig. 20, the expression of eleven chemokines among the fourteen investigated was identified. The lymphangioma was found to express chemokines belonging to all three subfamilies: C-X-C or α chemokines (KC, IP-10, MIP-2, BCL and Mig-1), C-C or β chemokines (Exodus, 6Ckine, RANTES, JE and C10) and C or γ chemokine (lymphotactin). Interestingly, three well known β chemokines (MIP-1α, MIP-1β and MIP-1γ) were not detected. These chemokine are secreted by haemopoietic lineage cells (monocytes, lymphocytes, neutrophils, mast cells), but also by fibroblasts, smooth muscle cells and tumour cell lines (300, 311, 323) MIP-1 α, β and γ display a wide range of biological activities. Among these activities remarkable is their role in different diseases such as rheumatoid arthritis and their HIV-suppressive effect as recently reported (53).

In most cases the correct identity of the amplified cDNA was also confirmed by sequencing (not shown). GAPDH fragment was amplified as internal control for each reaction.

To evaluate the relative expression level of each chemokine, the same amplified fragments were used as probes in Northern blot analysis of total lymphangioma RNA (Fig. 21). GAPDH was used as an internal control.

Positive expression as judged by a signal in the Northern blot was confirmed for most but not all the chemokines that were detected by RT/PCR analysis.

The hybridization signals of KC and RANTES molecules were not detectable even after a week long exposure, whereas all other bands shown in Fig. 21 were obtained after o.n. exposure. The identified signals corresponded to the expected sizes of mRNA of the different chemokines and revealed the existence of different expression levels among the investigated molecules.

Once again, positive chemokines belonging to the three different subfamilies were identified. Mig-1 was the α-chemokine with the highest expression level, while JE and C10 were the highest ones of the β subfamily. Lptn (γ chemokine) resulted weekly positive.

Fig. 20 shows amplified fragments corresponding to Exodus and 6Ckine. Although these molecules were described independently as two different chemokines (43, 123), their nucleotide sequences differ only by three nucleotides (position 38, 41 and 194 of the cds), suggesting that 6Ckine and Exodus represent a polymorphic version of the same molecule. The two 5' primers used ended at its 3' end at nucleotide 41 and therefore were specific for each polymorphic version. The high homology between the two sequences did not permit to discriminate the relative expression levels of both chemokines by Northern blot analysis since the unique signal represented the expression of both molecules.
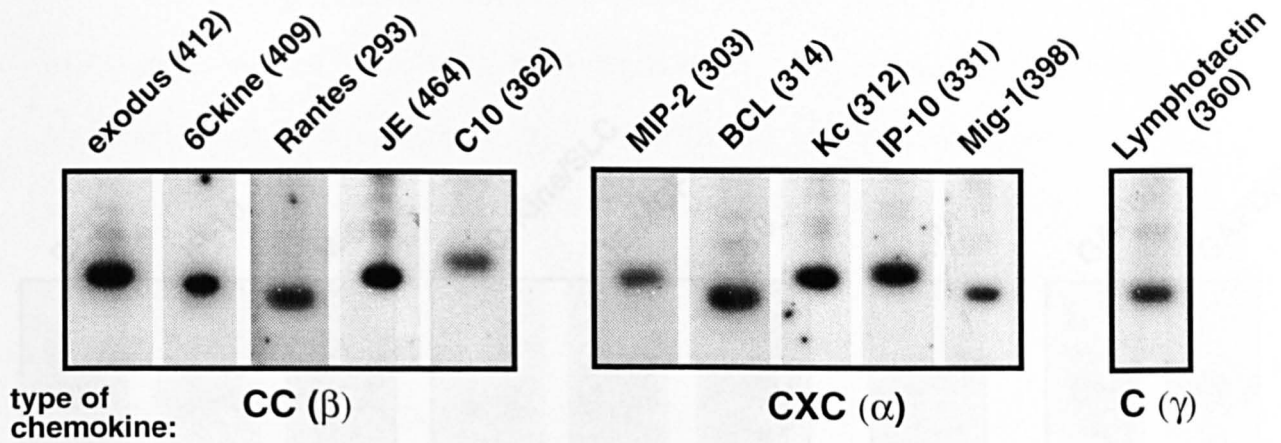
Figure 20. RT/PCR analysis of Chemokine Expression in Lymphangioma.

Each lane shows the autoradiogram of hybridization with a specific probe for the amplificated segment of mRNA, corresponding to the complete cds of the investigated chemokine. Sizes of the different amplificated are indicated in base pairs (bp) close to the molecule name. Chemokines of the same subfamily are grouped togheter.

type of
chemokine: **CXC (α)**   **CC (β)**   **C (γ)**
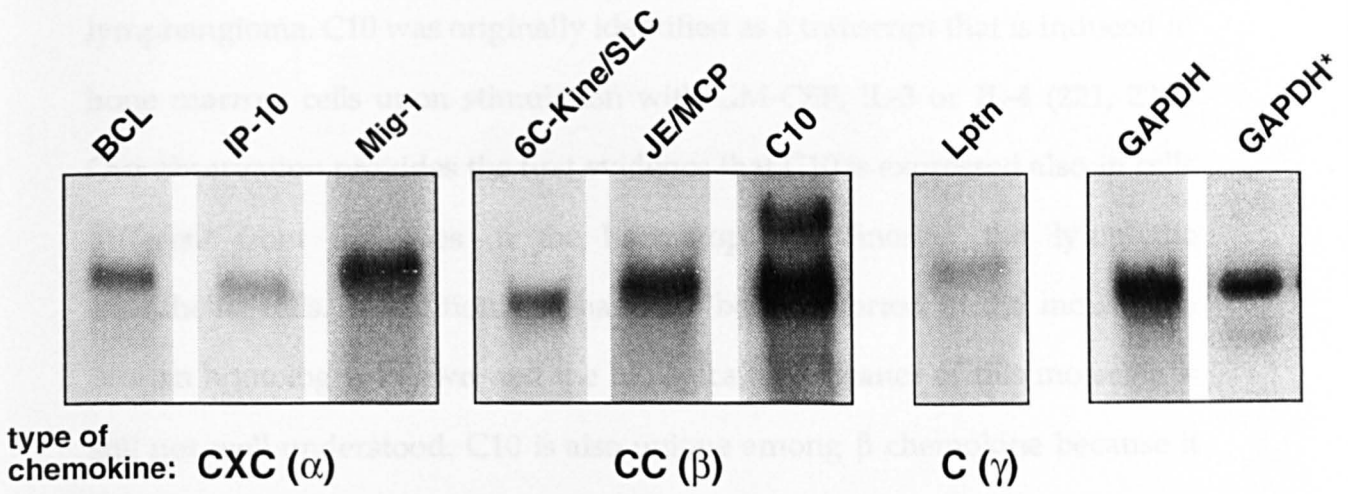
Figure 21. Northern blot analysis for Chemokine expression.

Northern blot analysis on total lymphangioma RNA showing different levels of expression of the identified chemokines. For each chemokine transcript the corresponding amplified fragment [α-^{32}P] dCTP labelled was used as hybridization probe. The autoradiograms shown in the figure were obtained after o.n. exposure. GAPDH was used as an internal control. Expression of KC and RANTES chemokines, identified by RT/PCR, was not confirmed by Northern blot analysis, while remarcable is the expression of C10. Total RNA of murine skeletal muscle cells was used as negative control: the line indicated by GAPDH* represents the expression in this sample. No chemokines expression was identified in murine skeletal muscle cells (data not shown).

GAPDH expression was identified in skeletal muscle cells mRNA, but no chemokines expression was revealed in the control sample (data not shown).

Interestingly, the signal of chemokine C10 detected in the Northern blots indicated an extremely high expression of this molecule by the lymphangioma. C10 was originally identified as a transcript that is induced in bone marrow cells upon stimulation with GM-CSF, IL-3 or IL-4 (221, 222). Our observation provides the first evidence that C10 is expressed also in cells different from the ones of the haematopoietic lineage: the lymphatic endothelial cells. In addition C10 has only been reported in the mouse, no human homolog is known and the biological significance of this molecule is still not well understood. C10 is also unique among β chemokine because it contains an extra second exon encoding a 16 amino acid sequence which is inserted in the amino terminal region of the protein. The sequence encoded by this exon plays probably a key role in recognizing and activating its specific receptor, that has not yet been identified (23). Because of all these reasons, the C10 chemokine was taken into consideration for further investigation.

3.9 In situ hybridization

All the information provided by the Northern blots analysis as well as those reported by chemotactic assay performed with the primary culture supernatants, were in accordance with the hypothesis that chemokines secretion was a functional property of the lymphangioma forming cells.

However, in order to assure that chemokines production by lymphatic endothelial cells was a general characteristic of the most of the tumour cells, *in situ* hybridization was performed on lymphangioma sections using specific anti-sense and sense RNA probes (Fig. 22).

Sequential tissue slides of lymphangioma were obtained from the same histological preparation that was used to perform the *in situ* hybridizations for Flt-4 and ICAM-1 above described (Fig. 17b, c).

For each chemokine subfamily we chose a molecule that in the previous assay showed a high level of expression: Mig-1, JE and lptn respectively corresponding to the α, β and γ subfamily were selected. *In situ* hybridization with C10 sense and anti-sense probe was also performed because the peculiarity of this C-C chemokine.

All anti-sense probes showed the same pattern of hybridization, restricted to the areas in which the lymphangioma lesion was present. Among the lymphangioma cells the staining intensity was variable, as previously also described for Flt-4 and ICAM-1. That could be due to different expression levels or to a variable accessibility of the probes to the cellular transcripts.

The liver parenchyma, cells of the diaphragm as well as vascular endothelial cells present in venules or in capillary structures resulted negative for hybridization with all the tested probes.

The same positivity detected in lymphangioma cells, was also observed in lymphonodes (Fig. 23). This finding suggests that endothelial cells of lymphonodes (HEV, high endothelial venules), and lymphatic endothelial cells of the lymphangioma, share a simile profile of chemokine expression.

	Antisense	**Sense**
Mig		
Je		
Lptn		
C10		
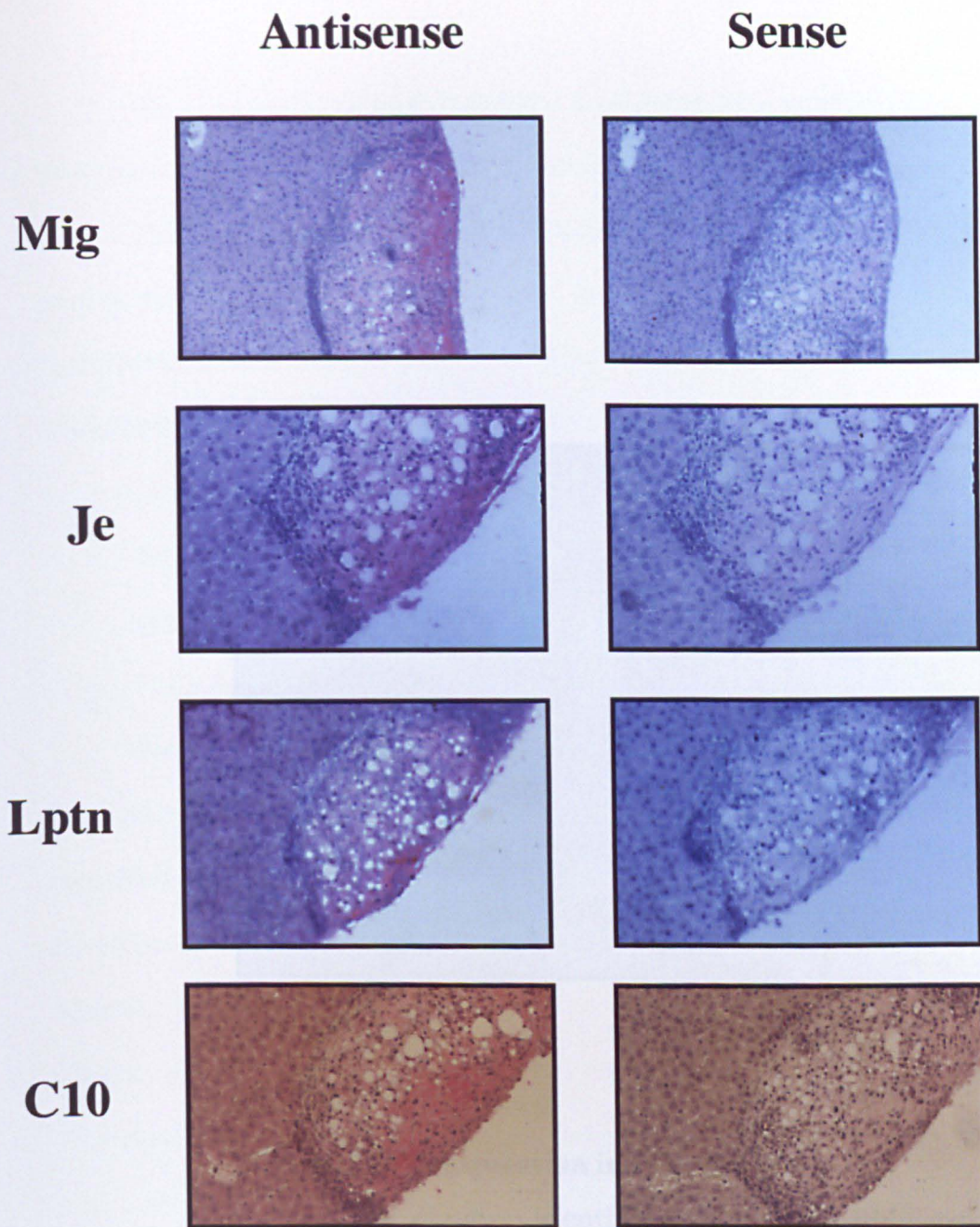
Figure 22. Analysis of chemokine expression in lymphangioma by In situ hybridization .

Paraffin embedded sections of lymphangioma were hybridized with digoxigenin-labelled sense and antisense RNA probes for Mig-1 (CXC chemokine), JE (CC chemokine) and Lptn (C chemokine). Because of its remarcable expression observed in Northern blot analysis, *in situ* hybridization was performed also for C10. The procedure was performed as indicated. All anti sense probes showed the same pattern of hybridization, restricted to the area in which lymphangioma is developed The same area was positive also for ICAM-1 and Flt-4 (Fig. 15b and 17b,c).

Figure 23. C10 expression in lymphnodes.
In situ hybridization identification of C10 mRNA expression in lymphnode. Paraffin embedded section hybridized with digoxigenin-labelled sense RNA probes for C10. Arrows indicate areas in which the staining is particularly intense.

As expected, control hybridizations with the corresponding sense RNA probes did not produce any detectable signal.

Taken together all the results obtained led to the conclusion that the leukocytes recruitment observed in the tissue surrounding the lymphangioma lesion is due to a functional property of the lymphatic endothelial cells: the secretion of a large number of different chemokines.

3.10 Chemotactic assay on different murine haemopoietic subpopulations

Supported by the molecular data and from the evidence that the lymphangioma derived cells conditioned medium (LCM) displayed a remarkable chemoattractant activity on human PMN (Fig. 19), it was considered more appropriate to test activity of the LCM in a homologous system. In order to characterize the supernatant's activity, chemotactic assays were performed using as target cells purified haemopoietic subpopulations: PMN, lymphocytes and monocytes/macrophage cells.

In addition, since we had observed an unexpected expression of the C10 chemokine in lymphangioma cells, previously described only as a product of bone marrow cells of the haemopoietic lineage (221, 222), we decided to investigate its activity. For this purpose, the eukaryotic expression vector pcDNA3 carrying the C10 complete coding sequence, was constructed and used to produce stable CHO transfectant clones expressing the C10 chemokine. Positive clones were identified by western blot analysis (Fig. 24) and their supernatants used in the chemotactic assays.
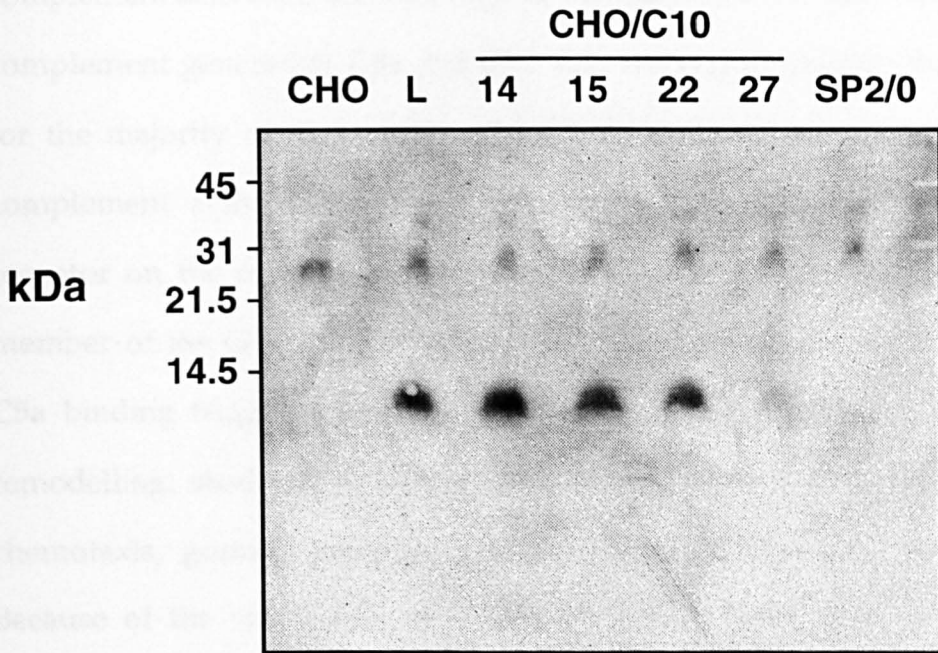
Figure 24. Western Immunoblot of C10 .

Detection of C10 protein secreted by CHO cells transfected with PCDNA3/C10 cDNA. 50 µl of differents supernatants of G418 selected clones (n. 14, n. 15, n. 22, n. 27) were tested (lanes 4-6) and the amount of C10 was compared with the one of 50 µl of lymphangioma conditioned medium. (L, lane 2). CHO untransfected cells supernatant (lane 1) and SP2O myeloma cell line conditioned medium (lane 7) were used as control.

C10 chemokine (approx. 10kDa), was detected with a goat anti-mouse C10. The reaction was developed by ECL.

Since cells of murine origin are very weakly responsive to human IL-8 chemotactic activity (276), medium containing 1% zymosan activated serum (ZAS) and recombinant murine MIP-1α (25 ng/ml) were used as positive controls. Zymosan is a precipitate of yeast cell walls obtained by zymolyase enzyme digestion. The incubation of serum with yeast cell walls induces complement activation and cleavage of the third and the fifth component of complement generating C3a and C5a. C5a is the glycoprotein that accounts for the majority of biologically relevant chemotactic activity produced by complement activation (266). It was shown that C5a bind to a specific receptor on the surface of human neutrophils (49). This receptor is also a member of the G-coupled seven transmembrane receptor superfamily and C5a binding trigger a number of complex effects including cytoskeletal remodelling, shedding of selectin and upregulation of adhesion molecules, chemotaxis, granule secretion and activation of NADPH oxidase (105). Because of the similarities of C5a/ZAS activity with that of chemokine molecules and their receptors, it is widely used as a positive control in chemotactic assays.

Chemotactic assay on peripheral blood PMN

Fig. 25 shows the data obtained from five independent experiments in which chemotactic activity of LCM, or C10-transfected CHO cells supernatant and related controls were tested on a cell preparation composed by more than 95% of peripheral blood neutrophils. After 30 min. of incubation, approximately 20% of neutrophils stimulated by LCM was able to transmigrate into the lower chamber. When stimulated by ZAS the

Mouse peripheral blood PMNs
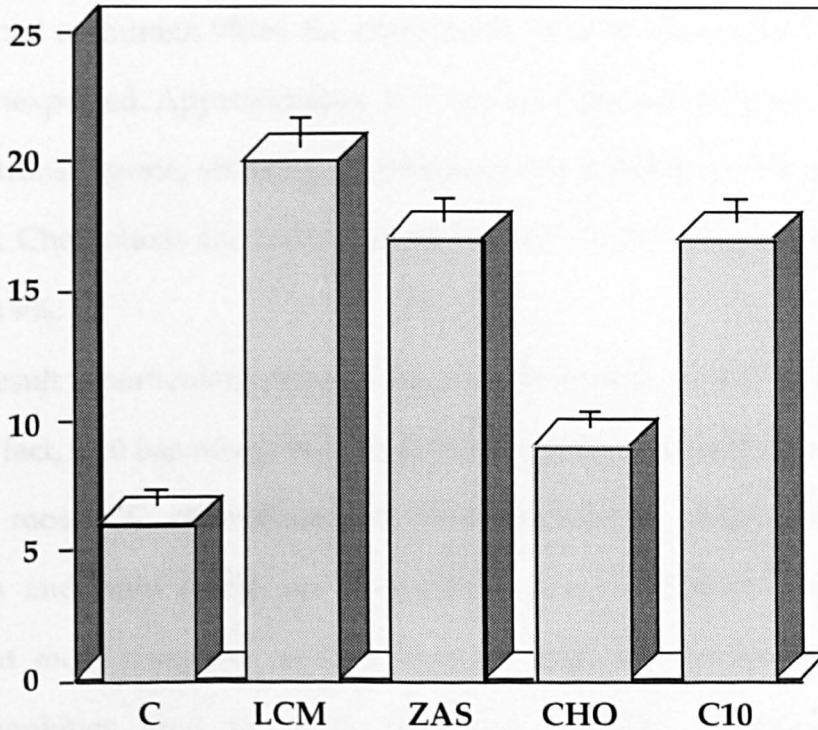
Figure 25. Chemotactic assay on mouse peripheral blood PMN.

PMN from mouse peripheral blood were purified on discontinuous Percoll gradient to >98% purity and used for chemotaxis with LCM (lymphangioma derived cells conditioned medium) and C10 in transwell chambers (pore size 1.2 μm). ZAS (Zymosan activated serum) was used as a positive control. Unconditioned medium of lymphangioma (C) and the supernatant of untransfected CHO cells are the negative control for LCM and C10 respectively. Results of five independent experiments are shown. The efficency of migration is expressed as a percentage of input cells migrating after 30 min. at 37°C. The means for LCM and C10 were significantly different from the controls. (p<0.001).

observed transmigration was 17% and was reduced to 6% in presence of the corresponding unconditioned medium. Thus, it appears evident that lymphangioma derived cells display a considerable chemoattractant activity also on these target cells. Although this data was expected because of the results obtained on human PMN, the chemotactic activity shown by C10 was somewhat unexpected. Approximately 17% of neutrophils stimulated by C10 was able to transmigrate, showing an efficiency comparable to the positive control ZAS. Chemotaxis by control untransfected CHO cells conditioned medium was 9%.

This result is particularly interesting mainly because of the target cells involved. In fact, C10 has never been shown to be active on neutrophils and, in addition, most CC chemokines act preferentially on monocytes and lymphocytes and only rarely on neutrophils. From different studies, it emerges that most receptors recognize more than one chemokine, and several chemokines bind to more than one receptor, indicating that versatility and promiscuity characterize the chemokine system. At present C10 receptor has not yet been identified, and therefore C10 may bind to receptors expressed on the surface of neutrophils. Furthermore, the regulation of C10 expression differs from the one reported for other molecules of the same subfamily, thus suggesting a distinct role for this molecule, even though its biological significance remains unclear.

Chemotactic assay on peritoneal PMN

The chemotactic activity of LCM and C10 was also evaluated on peritoneal neutrophils. These cells differ from their counterpart present in

95

Mouse peritoneal PMNs
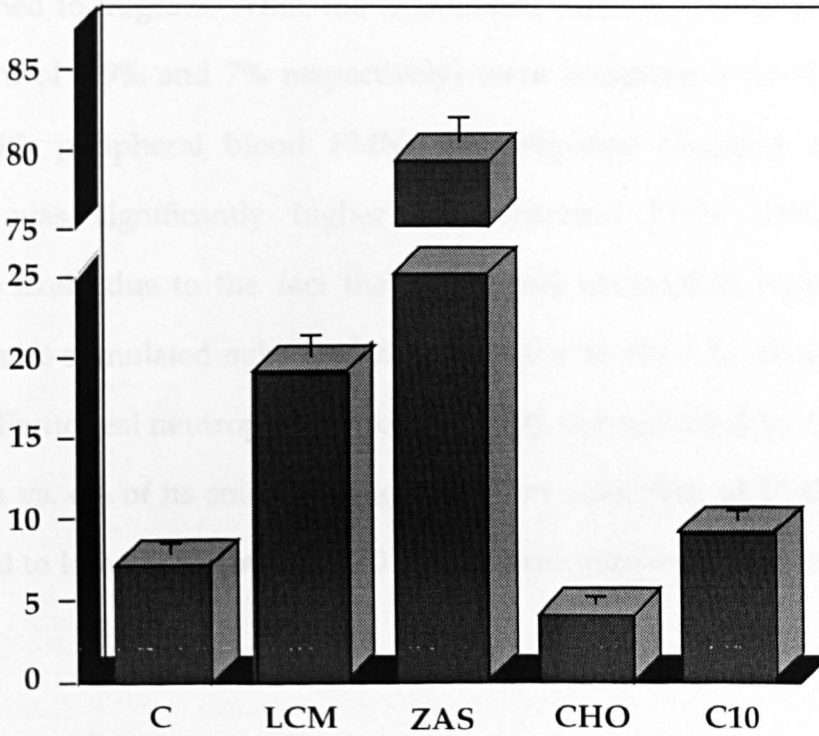
Figure 26. Chemotactic assay on mouse peritoneal PMN. Polymorphonuclear cells from peritoneum were isolated from the peritoneal exudate of a thioglycolate peritoneal injected mice and separated on discontinuous Percoll gradient to >95% purity. Peritoneal PMN were tested for chemotaxis to LCM and C10 in transwell chambers (pore size 1.2 µm). ZAS (Zymosan activated serum) was used as a positive control as described. Unconditioned medium of lymphangioma (C) and the supernatant of untransfected CHO cells are the negative control for LCM and C10 respectively. Histograms indicate the medians of four independent experiments . (p<0.001).

peripheral blood because of their level of activation. The recruitment of neutrophils from blood vessels into the peritoneal area is induced by the presence of thioglycolate in the peritoneum.

The figure 26 shows the results obtained on these PMN that are already primed to migrate. While the chemotactic activities of the LCM and negative control (19% and 7% respectively) were comparable to the ones obtained with peripheral blood PMN, the response obtained to ZAS stimulation was significantly higher on peritoneal PMN (78%). This difference is likely due to the fact that peritoneal neutrophils represent a selected, *in vivo*-stimulated subpopulation sensitive to the C5a chemotactic effect (276). Peritoneal neutrophils were practically not recruited by C10 (9% of migration vs. 4% of its control) suggesting that activation of PMN could be associated to loss of response to C10 possibly through down regulation of its receptor.

Chemotactic assay on lymphocytes

In order to evaluate the chemoattractant activity of LCM on murine lymphocytes, the assays were performed with an incubation time of 3h and using MIP-1α as positive control. In general lymphocytes have a reduced migration capacity compared to neutrophils and monocytes. Differences of approximately 10% of migration are considered an accepted criteria to establish positivity in a migration assay of lymphocytes. As shown in Fig. 27, LCM and C10 were active when tested on a population composed by more than 95% of lymphocytes. More than 22% of cells incubated with LCM vs.

Mouse peripheral blood lymphocytes
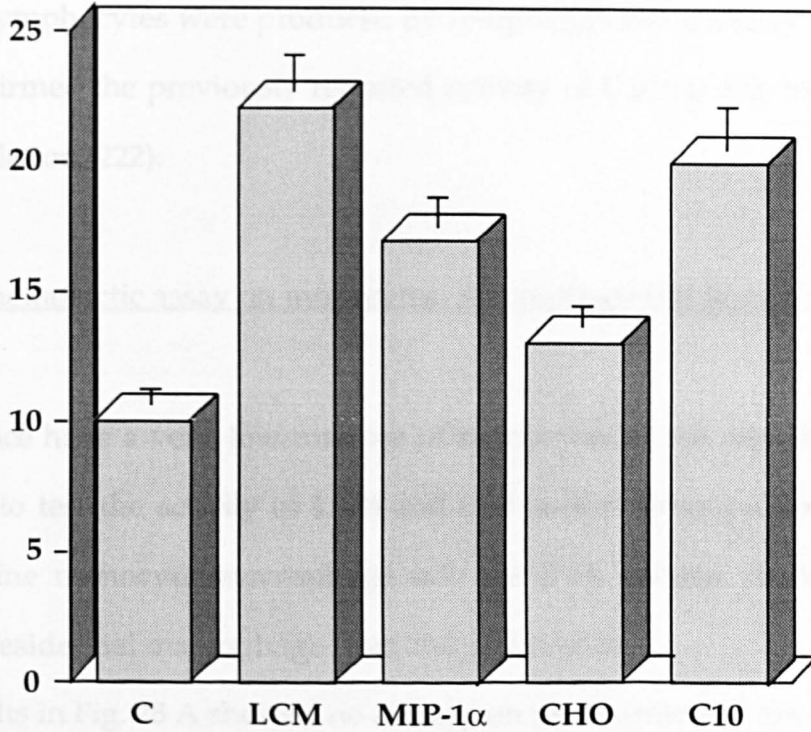
Figure 27. Chemotactic assay on mouse peripheral blood lymphocytes.

Mouse lymphocytes were separated on discontinuous Percoll gradient to >95% purity and tested for chemotaxis to LCM and C10 in transwell chambers (pore size 1.2 μm). MIP-1α (25 ng/ml) was used as a positive control. Unconditioned medium of lymphangioma (C) and the supernatant of untransfected CHO cells are the negative control for LCM and C10 respectively. Histograms indicate the means of five independent experiments evaluated with the criteria previously described after 3hrs of incubation at 37°C. (p<0.001).

the 10% incubated with the unconditioned medium, was able to transmigrate whereas the MIP-1 α positive control showed an activity of only 17%.

Similarly, 20% of lymphocytes were recruited in the presence of C10 compared to 13% of its control. These result indicated that chemokines for murine lymphocytes were produced by lymphangioma primary culture and also confirmed the previously reported activity of C10 on this haemopoietic subpopulation (222).

Chemotactic assay on monocytes/macrophage cell lineage

Mice have a very low number of monocytes in the peripheral blood. In order to test the activity of LCM and C10 on this subpopulation we used the murine monocytic-macrophage cell line J774, human monocytes and murine residential macrophage from the peritoneum.

The results in Fig. 28 A showed no activity on the murine cell line (even after repeated independent preparation of LCM), while positive chemotaxis was obtained on human cells (Fig. 28 B).

The murine peritoneal derived macrophages, which represent a more differentiated subpopulation of monocytes were also used as an alternative source of cells for the chemotactic assay. In Fig. 28 C are recorded the data obtained in assays performed with these cells. It is clear that LCM, was able to induce 60% of macrophage migration, comparable to the efficiency shown by the MIP-1α and ZAS positive controls (50% and 70% respectively). These results are indicative of the natural predisposition of activated peritoneal macrophage for migration.
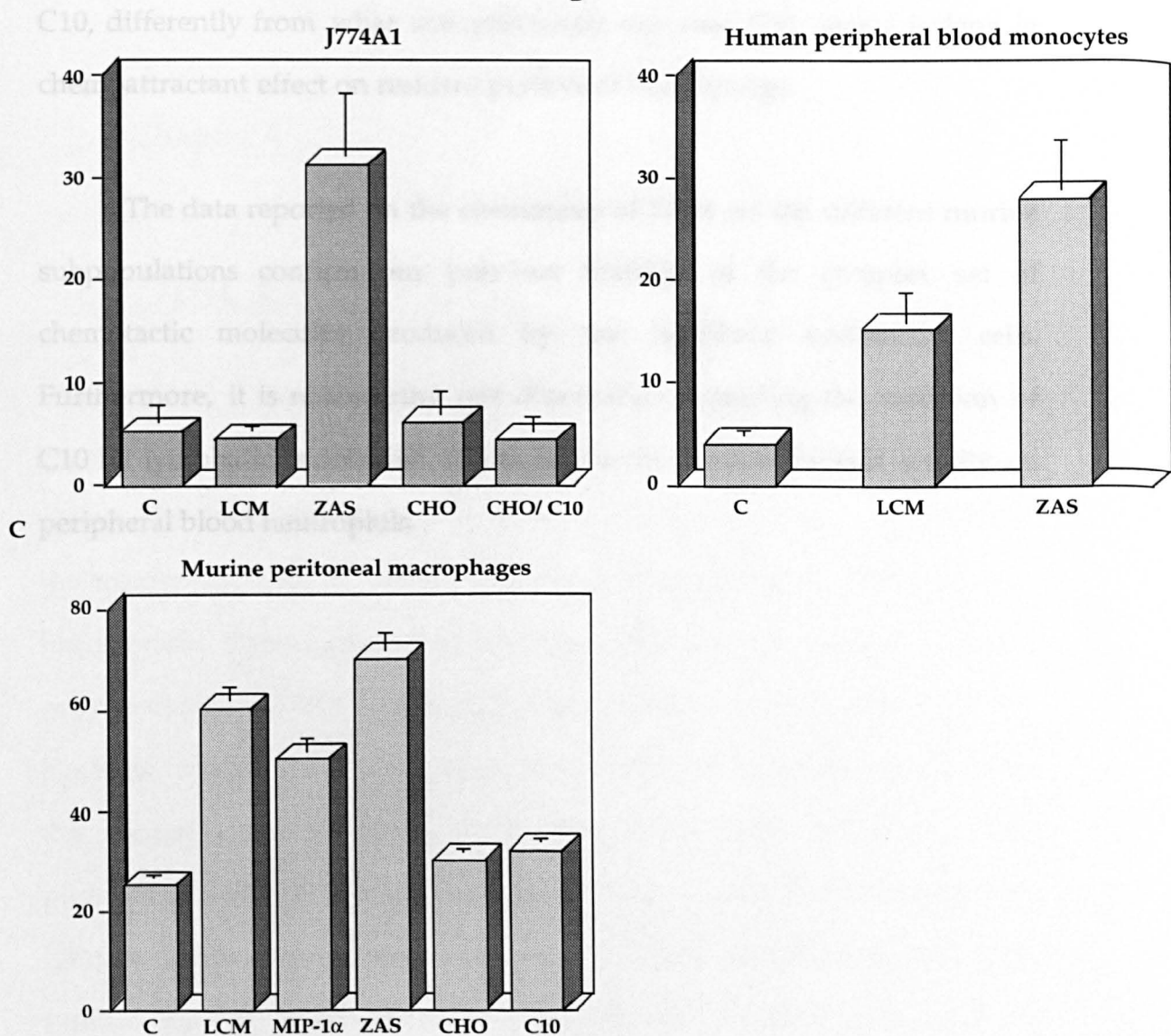
Figure 28. Chemotactic assay on monocyte/macrophage cells.

Chemotactic assays for cells belonging to the monocyte/macrophage lineage were performed in transwell chambers in which the pore size of the filter was 5μm and migration time was 1h at 37°C. As positive control for monocyte/macrophage chemotaxis was used ZAS (Fig. 25 A, B, C), and MIP-1α (25 ng/ml) (Fig. 25 C). All the reported data are the means of several experiments and by paired T-test was evaluated the significance of the differences obtained. To establish, at the described condition, the chemotactic activity of LCM and of C10 on monocyte cells were used the murine monocytic cell line J774A.1 (A), and human peripheral blood monocytes (B). In fig. 25 C are indicated the percentage of migration of resident peritoneal murine macrophages.

C10, differently from what was previously reported (23), seems lacking in chemoattractant effect on resident peritoneal macrophage.

The data reported on the chemotaxis of LCM on the different murine subpopulations confirm our previous findings of the complex set of chemotactic molecules produced by the lymphatic endothelial cells. Furthermore, it is noteworthy our observation regarding the secretion of C10 by lymphatic endothelial cells as well as its chemoattractant activity on peripheral blood neutrophils.

Chapter 4

DISCUSSION

The understanding of the biology of angiogenesis is important for the comprehension of several normal and pathological processes, like haemostasis, thrombosis, atherosclerosis, inflammatory disorders and the neovascularization of tumours (92). The interest in angiogenesis, however, has been focused almost entirely on blood vessels and their endothelium. On the contrary, much less is known about the molecular biology and physiology of the lymphatic endothelium and the process of lymphangiogenesis in normal and pathological conditions. One of the reasons for this is the difficulty of establishing lymphatic endothelial cell cultures. Mouse lymphatic endothelial cells have been obtained from animals treated for long periods with the parasite *Brugia malayi* (160) or from the high endothelial venules (HEVs) of peripheral lymph nodes (25). Other reported examples are represented by the cultures of lymphatic endothelium isolated from bovine mesenteric lymphatic duct, from canine and human thoracic duct and from a patient with a massive cervicomediastinal lymphangioma (30, 108, 138, 166).

In humans, lymphangiomas are uncommon benign tumours, most frequently found in children in the head and neck and peritoneal regions,

and may also arise and enlarge rapidly in adulthood (170). The origin of this disordered lymphangiogenesis is still unclear and a congenital predisposition was suggested by different authors especially for the childhood forms (310). From clinical investigation and experimental evidences (59, 216, 46, 51) arose the hypothesis of a possible link between the pathophysiological events of lymph stasis and disturbed lymphangiogenesis. Anatomical or functional lymphatic obstruction leads to a heightened intralymphatic transmural pressure which is transmitted to the endothelial cells as mechanical stress. This shear/strain effect, then, signals the lymphatic endothelial cells to release angiogenic mediators, similar to those produced by blood vascular endothelium (57, 195) inducing cell proliferation. The subsequent response may further enhance lymph formation despite a reduced lymphatic transport capacity. The accumulation of protein-rich edema fluid progressively disrupts attachments and adhesion of endothelial cells, promoting lymphatic remodelling and collateral formation. The angiogenic process reduces progressively the lymphatic drainage, involves release of growth factors and alterations of the extracellular matrix. All the process culminates in a dysplastic soft tissue overgrowth and occasionally in a highly aggressive lymphangiosarcoma. Nevertheless, also in cases of aggressive lymphangiosarcoma reported in humans, the frequency of metastasis establishment from this tumour is extremely low.

Although experimental and spontaneous haemangiomas have been widely documented, this type of tumour has not been previously described in mice (124). Thus, the procedure we describe here is the first simple and rapid procedure for inducing lymphangiomas in mice with extremely high efficiency. The lymphangiomas obtained represent a unique and source of

lymphatic endothelial cells. We have shown that cells derived from this murine tumours can be expanded as primary cultures for several weeks leading to the spontaneous formation of vessel-like structures as was previously reported in lymphatic endothelial cell cultures from a human cervicomediastinal lymphangioma and in culture of cells of bovine thoracic duct origin (30, 168). This suggests that, although cells are derived from a tumour, they retain morphogenetic properties similar to normal cells. This possibly reflects the fact that the lymphangioma is a benign lesion with accumulation of cells of rather normal phenotype. Therefore the murine lymphangioma, could represent a convenient animal model to understand the basic mechanisms of lymphangiogenesis and functions of lymphatic endothelial cells as well as those related to disorders associated to these cells, such as lymphedema, angiofollicular hyperplasia, lymphangioma, lymphangiomyoma and malignant lymphangiosarcoma (309).

The murine lesions obtained with injection of incomplete Freund's adjuvant, developed after few days in the peritoneal area, in a localization similar to what has been described for human acute lymphangiomatosis when it does not develop in the mediastinal area. The histological examination of the murine tumours revealed striking morphological and hystochemical similarities with the human counterpart (80). The lesion appears to be composed predominantly of a type of cells at different stages of differentiation, forming more compact areas (the parenchymatic parts of the tumour) and areas with more evident formation of lumen like structures. The lesion is not infiltrating the surrounding tissues and, as expected in this benign hyperproliferative disorders there is not involvement of endothelial cells of vascular origin as well as fibroblasts. The lesions does not shows any

presence of a basal lamina. Based on these observations it was hypothesized the lymphatic endothelial origin of the lesion. This hypothesis was supported by the following observations: a) the tumours had white colour, contrary to what is observed in haemangiomas, b) no red blood cells were seen inside most of the vessels, c) cells contained cytoplasmic vesicles of various sizes that seem to enlarge progressively and fuse with vesicles of adjacent cells to form the characteristic lumens, e) lumen like structures in which are evident the nuclei belonging to the different cells that are delineating the area, suggesting that these cells have the potential capacity to form vessels.

The intraperitoneal administration of incomplete Freund's adjuvant has been widely used to induce myeloma in mice, to enhance an antibody response to antigens in vaccination protocols and to induce experimental immunological illnesses like arthritis and autoallergic disease (97, 236, 239,293). However it has not been previously associated with the induction of lymphangiomas, in several publications lesions were reported to be caused by Freund's adjuvant, when administered at the doses here described. The lesions included polyarthritis, granulomatous proliferations in various organs and lipogranulomas of the peritoneum (128). These lesions, particularly the lipogranuloma, might actually correspond to what we have now characterized as lymphangioma. A single report has associated proliferation of the lymphatic endothelium in mice with the injection of Freund's adjuvant in footpad (156).

The formation of the tumour is likely to be due to a direct stimulation of the peritoneal lymphatic vessels draining the oil adjuvant. This hypothesis was further supported by the observation that pristane, another

oil experimentally use in mice in order to favour the ascitic growth of myeloma and hybridoma cells (161) in the peritoneal cavity, was also effective in inducing the tumours. It may be possible that the density of these oils generate the lymph stasis that was suggested to be at the origin of the mechanism that leads to the disturbed lymphangiogenesis (46, 250). Moreover, the angiogenic potential of different lipids such as prostaglandins PGE_1, PGE_2, and certain uncharacterized polar lipids has also been described (93). Once induced, the lesions were permanent. Even 10 months after their induction, all animals showed tumours which were similar to those observed at the early stages, without any progressive development of the lesions. A neoplastic lesion was observed in the axillo lymph node in a single case over more than two hundred mice treated in this period. The benign nature of the lesion was also confirmed by the absence of cell proliferation and new tumours establishment when lymphangioma derived cells were injected in the peritoneal area of host syngenic mice. Certainly it will be extremely useful add extra experimental details (i.e. bromodeoxyurodine studies to examine cell proliferation within the lesion; apoptosis studies to look at the extent of programmed cell death within the masses) in order to address the question if the described lymphatic lesions are tumours or represent phenomena of hyperplasia. Nevertheless, according to the definition that swelling or organ enlargement can also result from inflammation, physiological (i.e. skeletal muscle) or pathological tissue adaptation and hypertrophy or hyperplasia are stimulus depending (287), and the lesions induced by Freund's adjuvant injections persist for long time after the initiating stimulus has been removed, this suggest that it may be correct think of the described lesions as a tumour of (lymphatic) endothelial cells.

In the work presented here we used different criteria to characterize the endothelial origin of the cells. We detected the expression of the endothelial markers PECAM-1/CD31, ICAM-1/CD54 and ICAM-2/CD102, as well as several endothelial cell growth factor receptors, like Flk-1, Flt-4, Tie-1 and Tie-2.

PECAM-1/CD31 is a cell-cell adhesion molecule expressed at very early stage in the vasculogenesis process and is maintained also in mature vessel because of its involvement in the formation of inter-endothelial junctions, which are important for lumen formation, cell polarity and vascular permeability specially in vessel of vascular origin (162, 244). The heterorganic distribution of PECAM-1 displayed in lymphangioma is probably due to the fact that not only its expression in endothelial cells of lymphatic vessels is commonly weaker than in the vascular one (291), but also the lesion is composed by cells characterized by an active level of proliferation.

In contrast, vWF was not expressed by the lymphangioma cells as shown by RT/PCR assay and no Weibel-Palade bodies were seen in electron microscopy. From data reported in literature the expression of vWF by the lymphatic endothelium is relatively controversial among different authors. Lymphatic endothelium of bovine thoracic duct has been shown to express vWF, but its expression became variable in human lymphoproliferative processes and it has also been shown to be absent in human lymph node HEVs (167, 168, 258). Probably, as widely described for the vascular counterpart, heterogeneity may be exhibited also by lymphatic endothelial cells. The other endothelial markers analysed, the integrins ligand ICAM-1

and ICAM-2, were both identified in lymphangioma lesion. The expression of ICAM-1 in endothelial cell is constitutively low, but its identification during the embryological process of vasculogenesis implies an early ontogeny for the endothelial signal transduction pathway necessary for leukocyte recruitment and suggests a developmental role for this member of the immunoglobulin superfamily (127). Moreover, ICAM-1 induction on endothelium by inflammatory cytokines may increase cell-cell interaction and leukocytes extravasion at inflammatory sites, whereas constitutive expression of ICAM-2 may be important for leukocyte trafficking in tissues, as in lymphocyte recirculation (62, 269).

The detected co-expression in lymphangioma of Flk-1 and its ligand VEGF, that is considered the most critical mediator of endothelial cell proliferation during embryonic vasculogenesis as well as in physiological and pathological angiogenesis in the adult (78, 87, 92) suggests an autocrine mechanism of growth stimulation as previously described for basic fibroblast growth factor (bFGF) (114). This co-expression is probably not sufficient to support by itself the rapid growth of the lesion, but supports the notion that tumour neovascularization as well as angioproliferative disease can be triggered by stimuli that induce endothelial cells to produce its own autocrine factor(s) (79, 231). Also the expression of VEGF-C, which is proposed as the molecule that may play a specialized role in the development of the lymphatic system (158), was investigated in lymphangioma, but without positive results. Probably VEGF-C and its receptor Flt-4 maintain the paracrine relationship that was suggested by the data obtained by embryological development studies.

Among all the endothelial specific receptors, Flt-4 is the most relevant one in the context of the lymphatic endothelium. Indeed, the Flt-4 tyrosine kinase receptor that has been identified at relatively early stage of mouse development (E.8.5) in the angioblast of head mesenchyme, in the cardinal vein and extraembryonally in the allantois, becomes restricted to developing lymphatic vessels during late stages of mouse embryogenesis (158). It has also been found in human cystic lymphangiomas and lymphangiomatosis (unpublished data of Taina Partanen and Kaipanen, 1995). The finding that Flt-4 is specifically expressed by adventitial cells is consistent with the view that also in mice this molecule represents a marker for lymphatic endothelial cells maintained in adult. We demonstrated by RT/PCR analysis, immunoblotting and *in situ* hybridization that the adjuvant-induced lymphangioma cells express Flt-4 and that the expression was also maintained in the *in vitro* propagated cell cultures producing the expected pattern of protein bands detected with the specific monoclonal antibody. In addition, the mice with a knock-in β–galactosidase gene in the Flt-4 locus gave specific staining for the reporter gene in part of the tumour cells. Unfortunately, the Flt-4/lacZ mouse was not available in order to follow the Flt-4 expression in cell culture and the immunocyto-chemistry performed with the only available antibody on the explanted tissue or on cultured cells, probably for its technical characteristic, failed to give the expected results. These data should be extremely informative to address the question about the possible heterogeneity of the cell population forming the lesion and the level of non-endothelial cells contamination at the time of a more "established" primary culture. At present, the available data will not exclude the possibility of cellular heterogeneity, nevertheless a new criteria was

recently used in order to prove the nature of the cells forming the described lesion. Recently, the podoplanin, a novel 43-kDa membrane protein of podocytes, was reported by Breiteneder-Galeff et al. (35, 306) as a new marker for lymphatic endothelium. RT/PCR analysis for podoplanin expression on explanted tumour as well as on cultured cells mRNA was performed obtaining a positive results (data not show). At present, an anti mouse-podoplanin antibody for further investigation, is not available, but these preliminary data strengthen the hypothesis that the tumour contains abundant lymphatic endothelium and may be considered an extremely convenient model for further investigation of the biological properties of these cells.

<center>*****</center>

The salient feature of a variety of inflammatory conditions, such as infection, allergic disorders, autoimmune diseases, or ischemia/reperfusion injury is the association of infiltrating leukocytes. These extravasating leukocytes often contribute to the pathogenesis of the underlying disease. However, it should be appreciated that leukocyte recruitment is also critical for the host defence, leading to clearance of the inciting factor(s), i.e. infection. While at present the events of leukocyte trafficking may appear intuitive, it has taken more than one century of research to elucidate in part the molecular and cellular steps involved in the process of leukocytes migration. The maintenance of leukocyte recruitment during inflammation requires intercellular communication between infiltrating leukocytes and the endothelium, resident stromal and parenchyma cells. These events are

mediated via the generation of early response cytokines, e.g. IL-1 , TNF-α, the expression of cell-surface adhesion molecules, and the production of chemotactic molecules, such as chemokines. Among the cell surface adhesion molecules, ICAM-1, which is expressed by activated endothelium and associates with the counter-receptor CD11a/CD18 (Leukocyte function antigen-1, LFA-1) expressed on polymorphonuclear cells, plays an important role in the mechanism of leukocytes transmigration during acute inflammation (179, 265) and becomes upregulated by several molecules such as IL-1, TNF-α, LPS (256). ICAM-1 upregulation promotes a dramatic increase in PMNs adhesion, with the participation of other accessory adhesion molecules of the selectin family like CD62E/E-Selectin and CD62P/ P-Selectin. Thus, this adhesion molecule is an inducible endothelial marker and its expression level is related to a functional state of the cells. The finding that LECs express ICAM-1 indicates that these cells represent an activated form of endothelium.

Over the past ten years, an increasing number of chemokines have been identified as attractants of different types of blood leukocytes to sites of infection and inflammation. However the information obtained led to the knowledge that leukocyte recruitment during inflammation is only one of the different functions displayed by chemokines. It has been shown that chemokines are also involved in the inhibition and stimulation of blood vessel formation (11), in leukocytes maturation in the bone marrow (293) and in the regulation of physiological lymphocyte traffic (26, 206). The continuous recirculation of lymphocytes through the blood, tissues and lymphatics in an organized manner brings naive lymphocytes into the

lymph nodes, where they encounter antigen and are transformed into memory lymphocytes.

Chemokines have been found to be secreted in response to a variety of factors by an array of cells including monocytes, alveolar macrophages, neutrophils, platelets, eosinophils, mast cells, T lymphocytes, NK cells, keratinocytes, mesangial cells, epithelial cells, fibroblast, smooth muscle cells, mesothelial and endothelial cells. The production of chemokines by both immune and non immune cells support the contention that chemokines play important roles in different processes. In order to investigate the chemokines biological effects, regulation of expression, and possible role in disease, different *in vitro* and *in vivo* approaches have been developed. In particular, the production of chemokine by endothelial cells in response to several stimuli (e.g. inflammatory cytokines, hypoxia, bacterial LPS) (185) is well documented. Most of the information, however, is related to the expression of chemokines by cells of vascular origin, while limited documentation exists in relation to lymphatic endothelial cells. This difference reflects the absence of suitable models for the isolation and the culture maintenance of endothelial cells of lymphatic vessels as well as the only relatively recent discovering of appropriate markers in order to characterize unequivocally these cells. The main model systems available for LECs consist on the use of high endothelial venules (HEV) and Peyer's patches cells which are rather heterogeneous in their composition and represent a very distinct stages of differentiation. The model of LECs isolated from lymphangioma that we describe offers the possibility of addressing other questions related to LECs functions. One of these is the production of chemokines. We became interested in this problem because of an

observation arising from the histological analysis of the lesion. It was evident the presence of leukocytes, mainly neutrophils, in the area of the tissue neighbouring the lymphangioma. In all the histological sections analyzed no infiltration of the recruited leukocytes into the lesion or any sign of necrosis in the area in which leukocytes accumulated was observed. These facts indicated that the lymphangioma was producing chemokines and suggested that it could represent a model for the characterization of the profile of chemokine expression by the lymphatic endothelium.

From the preliminary screening performed by RT/PCR analysis, arose the evidence that different chemokines were expressed by lymphatic endothelial cells forming the lymphangioma. With the exception of the MIP-1α, MIP-1β and MIP-1γ chemokines, known to be secreted mainly by the monocyte/macrophage cell lineage (311, 323), expression of the other chemokines, belonging to all chemokine subfamilies (CXC, CC and C) was detected. This finding suggests that the possible contamination by monocyte/macrophage cell lineage in the lesion induced by Freund's adjuvant could be relatively low, or that these cells, if present, are not activated, but this hypothesis seems rather improbable.

Among the detected molecules, the expression of CXC chemokines IP-10, MIG-1, BCL, and of CC chemokines Exodus/6Ckine, JE, C10 and the C chemokine Lymphotactin was confirmed by Northern blot and *in situ* hybridization. Some of these are novel chemokine (e.g. BCL, Exodus-2, 6Ckine) that are selectively expressed in secondary lymphoid organs (spleen, lymph nodes, thymus, Peyer's patches), attract preferentially T or B lymphocytes and could play a role in lymphocytes homing and maturation.

Interestingly the expression of KC, a CXC chemokine that together with MIP-2α and MIP-2β (218, 283, 311), performs the functions of IL-8 in mice, was not confirmed by Northern blot suggesting a very low level of expression. A similar situation was observed for RANTES which is a CC chemokine with potent chemotactic and activating properties for basophils, eosinophils and NK cells (141, 169). This chemokine is also known to have HIV-suppressive effect and to synergize with MIP-1α, MIP-1β in the suppression of HIV (53). Interestingly, these chemokines exhibit 30% amino acid sequence identity to the two genes product of Kaposi's Sarcoma associated herpes virus (KSHV or HHV-8) the etiological agent of the angioproliferative malignant lesion associated with HIV infection. At present there are no functional data on the viral CC chemokines, which could be involved in the chemoattraction of haemopoietic cells into KS lesions (197, 207)).

For all the other chemokines tested the demonstration of their expression in lymphangioma was established by *in situ* hybridization. The data obtained suggests a complex profile of chemokine expression by LECs which may include other members of the chemokine family that were not investigated here. In addition it is also possible that LECs express specific chemoattractant molecules. Previous reports have led to the general consensus that members of the CXC subfamily are chemotactic to polymorphonuclear leukocytes (PMN) including neutrophils, eosinophils an basophils, while members of the CC subfamily are generally chemotactic to mononuclear cells such as monocytes and lymphocytes (217). However some exceptions to this paradigm have been described in both CXC and CC

subfamilies KC, IP-10 and JE, RANTES respectively (13). The C chemokine Lymphotactin is chemotactic exclusively for T lymphocytes (149).

Among all the chemokines expressed by LECs, we found members of all three families. Although some of chemokines function on PMN, a large number of chemokines produced by LECs are specifically active on different populations of lymphocytes (Leptn, Exodus/6Ckine, BCL, IP-10, MIG).

A particular case is that of C10 chemokine. This molecule was previously described as a chemoattractant exclusively produced by murine bone marrow derived cells or resident peritoneal macrophages cells after stimulation with GM-CSF, IL-3 or IL-4 (221, 222). The biological significance of this chemokine is still unknown. Even though structurally it belongs to the CC chemokine subfamily, it has been reported to be active on mouse peritoneal exudate cells (PECs) as well as on human peripheral blood mononuclear cells (23, , 222).

Our results show that C10 was actively produced by LECs representing one of the chemokines with the highest level of expression and suggesting that it could play a relevant role in the chemoattractant activity of LECs for lymphocytes. Surprisingly, we observed that C10 was active on peripheral blood neutrophils, while it was not on peritoneal neutrophils, and residential peritoneal macrophages, two types of cells with high migrating capacity, differently from what has been reported (23) .

C10 differs from other CC chemokines RANTES, MIP-1α, and JE in its regulation. Although these chemokines are all induced by LPS treatment of bone marrow-derived macrophages and/or resident peritoneal macrophages, C10 stimulation by LPS was never observed. Conversely, C10 is strongly induced by IL-3 and GM-CSF in both macrophage populations,

whereas the induction of RANTES, MIP-1α, and JE not only is weaker, but also restricted to bone marrow-derived macrophages. Finally, IL-4 strongly induces C10 in a dose-dependent manner, while is ineffective for the other chemokines. IL-4 has been reported to display many effects that enhance macrophage function (6, 272, 292) and others that appear to be anti-inflammatory, such as suppression of interleukins production, down-regulation of macrophage cytotoxic and microbicidal activities, reduction of Fcγ receptor expression (122, 282, 134). The selective induction of C10 by IL-4 suggests that this chemokine plays a role in mediating these functions, in particular it might promote the cell-cell interactions involved in the development of humoral responses (222). Supporting this hypothesis, it has been reported that C10 is chemotactic for both B cells and CD4^{+}T helper cells, although details in the mechanism of action are still limited by the fact that the receptor of this chemokine is not yet known, neither the human homolog has been identified. In other cases, such as the murine CCF18 and the human MIP-1 delta (121, 301) differences in cell target activity and regulation have been described in spite of their homology and similar structural features.

As mentioned above, recruitment of PMNs was observed in the region surrounding the lesion. We found that two chemokines (JE and MIP-2) of well documented activity on these cells were expressed by LECs and could in part explain our observation. An interesting question is related to the absence of necrosis of the recruited cells as well as the absence of infiltration into the tumour. From studies on inflammation processes it is well known that neutrophils, or more generally granulocytes, leave the blood stream to migrate into the tissue following appropriate inflammatory

stimuli, but once arrived to the injured area, they perform their biological functions and die *in situ*. There is not a clear explanation for the absence of necrosis that we observed, neither for the strong recruitment of PMNs in the area proximal to the lesion, since these cells are not able to recirculate. One possibility is that other yet unidentified molecules produced by the LEC can control this process or that the observed event is not related to an inflammatory process.

Among the chemokine expressed by LECs we identified several molecules with known activity on lymphocytes. This was paralleled by the migration assay performed with the supernatant of LECs. Those results are particularly relevant because they represent the first direct evidence that lymphatic endothelial cells participate in the control of lymphocytes trafficking and homing, a process not only important for the establishment of specific defence in pathological situations, but also for the maintenance of the immunological homeostasis in a organism, through the production of chemokines. Following the hypothesis that the LECs obtained from the experimental induced lymphangioma, are able to secrete chemokines of various type, new questions arise. For instance, it is not yet clear which of the chemokines are actually expressed by normal LEC and which is the relative importance of each one of them in lymphocytes trafficking within lymphatic vessels. Some indications come from the results obtained with lymphangioma derived cells spontaneously transformed and kept in culture for prolonged time and from primary culture obtained from tumours harboured in mice for period longer than six months. When those cells were analyzed by RT/PCR, they were still positive for the expression of some

chemokines active on lymphocytes such as IP-10, lptn and MIG (data not shown).

The possibility to identify new chemokines restricted to lymphatic endothelium expression would be extremely helpful for the understanding of the physiological recirculation of lymphocytes, and could provide new lymphatic endothelium specific markers.

Independently from the evidences reported by the presented data, many shadow areas remain to be enlightened and questions to be addressed. Up to date several attempts were done to immortalize a cell line Flt-4 and podoplanin positive starting from LECs. So far these attempts were unsuccessful, but recently a spontaneous transformed clone, already tested by RT/PCR analysis, seems to present these characteristics. The opportunity to work with a homogeneous lymphatic endothelial cells line, will be extremely promising to answer many of the questions arising from present thesis, first of all the possibility of demonstrate the origin of the chemokines produced, as well as to determine which stimuli induce chemokines expression and their relative/reciprocal regulation.

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