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Endothelial Cells and Cancer

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Abstract Endothelial cells play a key role in the development and function of blood and lymph vessels. Excessive proliferation and transformation of endothelial cells lead to pathological angiogenesis/lymphangiogenesis or vascular malfunctions which are hallmarks of malignant disorders. There is emerging evidence that circulating endothelial progenitor cells (EPCs) also contribute significantly to these processes. Major progress has been achieved over the past few years in the identification of key molecules involved, and in targeting tumour angiogenesis for human therapy. Current research efforts are concentrated on deciphering the origin and functional properties of endothelium in various tumours, as well as endothelial neoplasms themselves. The aim of these studies is to investigate the molecular mechanisms regulating mobilisation of EPCs from bone marrow, and their homing and differentiation into mature endothelium in situ at sites of neovascularisation, as well as the role of viral oncogenes in regulating the plasticity and extending the life span of endothelial cells. Integrated understanding of the mechanisms regulating the properties and function of endothelial cells during tumourigenesis is resulting in the development of a number of exciting and bold approaches for the treatment of cancer.

Keywords Endothelium · Cancer · Neoplasm · Angiogenesis

1 Introduction

1.1 Endothelial Cells: Role in Vascular Development and Function, and Impact on Health

The growth of new blood and lymph vessels is required for embryonic development and stimulates the healing of injured tissues; however, it also promotes tumour growth and inflammatory diseases (Carmeliet 2003). Endothelial cells play a key role in neovascularisation. Blood and lymphatic capillaries consist of thin-walled vessels composed of a single layer of endothelial cells (microvascular endothelium), and larger vessels are surrounded by mural cells (pericytes in medium-sized vessels, or smooth muscle cells in large vessels). New endothelial cells originate through differentiation from endothelial progenitor cells (EPCs) or proliferation of mature endothelium in pre-existing vessels. In a normal adult, most vasculature is quiescent, with only 0.01% of endothelial

Table 1 Angiogenesis and lymphangiogenesis in endothelial and non-endothelial neoplasms

System/organ	Processes characterised by abnormal angiogenesis/lymphangiogenesis*
Reproductive system (uterus, ovary, breast)	Neoplasms[a,b]
Bone, joints	Neoplasms[a,b]
Liver, kidney, lung and other epithelia	Neoplasms[a,b]
Brain, nerves, eye	Neoplasms[a,b]
Skin	Neoplasms[a]
	Kaposi's sarcoma[a,d]
Blood vessels	Atherosclerosis[a]
	Haemangioma[c]
	Haemangioendothelioma[c]
Lymph vessels	Tumour metastasis[b]
	Lymphoproliferative disorders[b,c]
	(e.g. lymphangioma, lymphangiosarcoma)
Haematopoiesis	Kaposi's sarcoma[a,d]
	Haematological malignancies[d]

*List of selected examples [a]Increased vascularisation due to abnormal angiogenesis [b]Increased lymphangiogenesis or derailed growth of lymphatic endothelium [c]Endothelial cell hyperplasia [d]Abnormal differentiation of progenitor cells or reprogramming of endothelial cells

cells undergoing division. Quiescent vascular and lymphatic endothelial cells are activated by (lymph)angiogenic factors such as various members of the vascular endothelial growth factor (VEGF) family, which stimulate endothelial cell proliferation and migration, thereby promoting new vessel formation (Alitalo et al. 2005; Ferrara 2004). Recent evidence suggests that EPCs also contribute significantly to de novo vessel formation during wound healing, limb ischaemia, post-myocardial infarction and endothelialisation of vascular grafts. However, excessive proliferation of endothelial progenitor or mature differentiated endothelial cells contributes to numerous neoplastic and non-neoplastic disorders (Table 1). For example, the growth of the majority of human tumours is accompanied by an increase in the number of proliferating endothelial cells and in vascularity. The transformation of endothelial cells is also implicated in the pathogenesis of Kaposi's sarcoma and other neoplasms of blood and lymph vessels. Current research is aimed at identifying the molecular mechanisms regulating these processes to enable effective targeting of endothelium for human cancer therapy.

2
Endothelial Progenitor Cells and Cancer

For many years, the prevailing dogma stated that vessels in the embryo developed from endothelial progenitors, whereas sprouting of vessels in the adult resulted only from division of differentiated endothelial cells (Fig. 1). Recent evidence, however, indicates that EPCs also contribute to vessel growth in ischaemic, inflamed or malignant tissues in the adult (Asahara and Isner 2002; Luttun and Carmeliet 2003; Rafii et al. 2002).

2.1
Endothelial Progenitor Cell Plasticity and Differentiation into Mature Endothelial Cell Sub-lineages

Endothelial and haematopoietic cells share a common progenitor, putatively termed the*"haemangioblast"* (Fig. 2). Both share certain antigenic determinants, including Flk-1 (vascular endothelial growth factor receptor 2, VEGFR-2), Tie-2, c-Kit (CD117), AC133 (CD133) and CD34.

During embryonic development, haemangioblasts in the yolk sac, in the arterial wall of the aorta and in the aorta-gonadal-mesonephros region form aggregates (blood islands) in which the inner cells develop into haematopoietic precursors and the outer population into endothelial cells. Angioblasts migrate extensively before in situ differentiation and plexus formation. VEGF, VEGFR-2 and basic fibroblast growth factor (bFGF) influence angioblast differentiation (Carmeliet et al. 1996; Carmeliet and Collen 1999; Ferrara et al. 1996; Shalaby

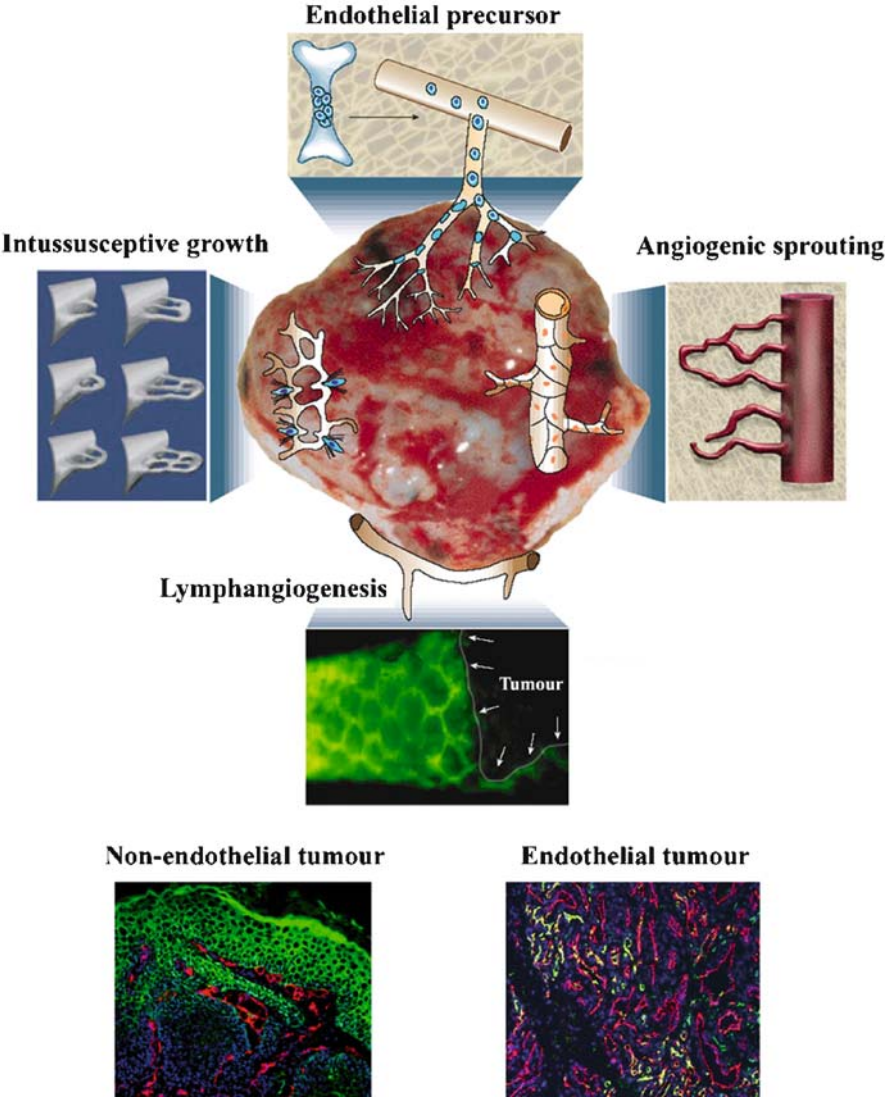
et al. 1997), whereas VEGFR-1 suppresses haemangioblast commitment (Fong et al. 1999). Molecules mediating interactions between endothelial cells and matrix macromolecules, fibronectin or matrix receptors (integrins) also affect vasculogenesis (Koch et al. 1995; Lyden et al. 1999; Table 2).

In the adult, EPCs are a unique cell population existing in peripheral blood mononuclear cells (PBMNCs), being derived from the bone marrow (BM) (Iwami et al. 2004). These cells incorporate into sites of active tumour neovascularisation, but also after ischaemic events in limb and myocardium, and thus contribute to de novo vessel formation (Asahara and Isner 2002). More recent studies have introduced the concept that the origin of EPCs may not be limited to the BM, e.g. tissue-specific stem/progenitor cells possibly provide in situ cells as another source of EPCs (Tamaki et al. 2002). Irrespective of origin, EPCs are mobilised and capable of homing to sites of neovascularisation under the influence of appropriate cytokines and growth factors, including VEGF as a critical molecule for vasculogenesis and angiogenesis (Asahara et al. 1999). Increased circulating VEGF promotes the mobilisation of EPCs from BM, resulting in increased numbers of circulating EPCs (Asahara et al. 1999; Hattori et al. 2001). Similar modulation of EPC kinetics resulting in their mobilisation to sites of neovascularisation has been observed in response to other haematopoietic stimulators, such as granulocyte-macrophage colony stimulating factor (GM-CSF) (Takahashi et al. 1999), angiopoietin (Ang-1; Hattori et al. 2001), stroma-derived factor (SDF)-1 (Yamaguchi et al. 2003), and erythropoietin (Heeschen et al. 2003). Rafii and colleagues proposed that mobilisation of EPCs from the BM requires angiogenic factor-mediated activation of matrix metalloproteinase (MMP)-9, which leads to the release of the soluble Kit ligand (Heissig et al. 2002). This ligand would in turn pro-

Fig. 1 Cellular mechanisms of tumour (lymph)angiogenesis. Tumour vessels grow by various mechanisms: (1) the host vascular network expands by budding of endothelial sprouts or formation of bridges (angiogenesis); (2) tumour vessels remodel and expand by the insertion of interstitial tissue columns into the lumen of pre-existing vessels (intussusception); and (3) endothelial cell precursors (angioblasts) home from the bone marrow or peripheral blood into tumours and contribute to the endothelial lining of tumour vessels (vasculogenesis). Lymphatic vessels around tumours drain the interstitial fluid and provide a gateway for metastasising tumour cells. Newly developed blood and lymphatic vasculature contribute to the development of both endothelial (e.g. angiomas and lymphangiomas) and non-endothelial (e.g. epithelial tumours) neoplasms. In non-endothelial tumours, endothelial cells (immunostained with panvascular endothelial marker CD31; *red*) contribute to skin cancer tumour angiogenesis [epithelial tumour cells express green fluorescent protein (GFP) construct under the control of keratin 14 promoter; *green*]. Endothelial tumours are mainly formed of blood or lymph vessels (i.e. endothelium) with a minor proportion of supporting tissue [endothelial origin of cells is revealed by double immunofluorescence with panvascular endothelial marker CD31 (*red*) and lymphatic endothelium-specific marker LYVE-1]. (Adapted from Carmeliet and Jain 2000; Hirakawa et al. 2005; Dadras et al. 2004)

mote proliferation and motility of EPCs within the BM microenvironment, thus creating permissive conditions for their mobilisation into the peripheral circulation.

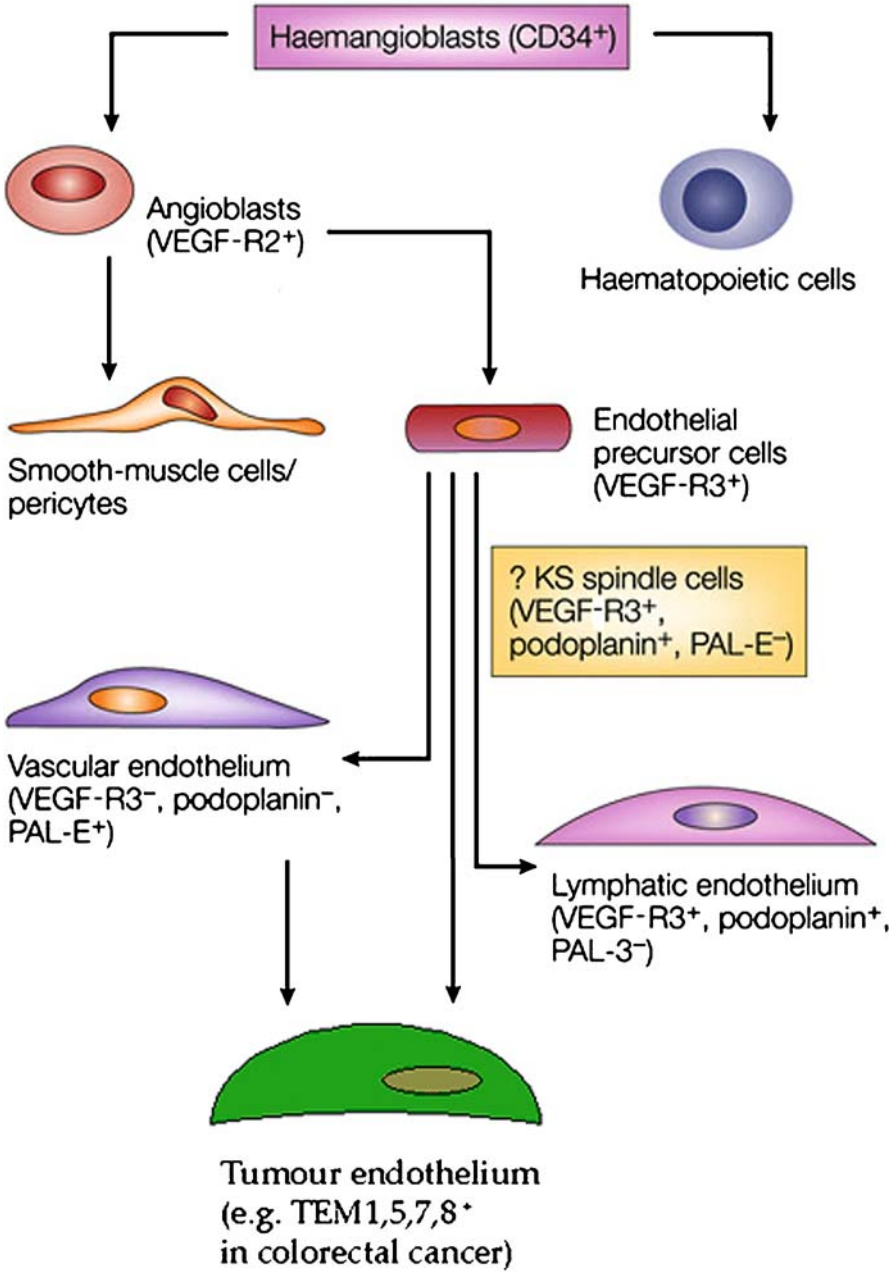
In situ (at the sites of neovascularisation), EPCs differentiate to endothelial lineage cells (mature endothelial cells), but not all endothelial cells are alike. Heterogeneity of differentiated endothelial cells depends on commitment of EPCs to produce arterial, venous or lymphatic endothelium, indicating that EC progenitors have remarkable *phenotypic plasticity* (Fig. 2). Recent genetic



studies offer insight into the signals controlling arterial and venous identities of endothelial cells, but little is known about the various pathways specifying them. For examples, the Notch pathway, with its ligands (Delta-like-4, Jagged-1 and Jagged-2) and receptors (Notch-1, Notch-2 and Notch-3), promotes the arterial fate of endothelial cells by repressing venous differentiation (Lawson et al. 2001; Zhong et al. 2001). Sonic Hedgehog and VEGF act upstream, whereas the bHLH (basic helix-loop-helix) transcription factor Gridlock probably acts downstream of Notch to determine arterial fate, even before the onset of flow (Lawson et al. 2001; Zhong et al. 2001). Ephrin-B2 and its receptor EphB4 are involved in establishing arterial versus venous identity, perhaps by fusing arterial and venous vessels at their junctions (Yancopoulos et al. 2000; Table 2). Furthermore, the molecular profile (transcriptome and proteome) of microvascular

Fig. 2 Putative pathways of normal and tumour endothelial cell differentiation. Endothelial cells in non-endothelial and endothelial tumours derive from either pre-existing normal vessels or from endothelial progenitors (see also Fig. 1). Normal endothelial cells are derived from progenitor cells (haemangioblasts) that can give rise to both endothelial and blood cells. Vascular endothelial growth factor (VEGF) family members stimulate these cells to develop into endothelial cells. The VEGF receptor-2 (VEGFR-2 or Flk-1) can be used to identify progenitors of the endothelial lineage (angioblasts). Angioblasts can give rise to smooth-muscle cells and pericytes, which form the outer walls of most blood vessels, or to endothelial precursor cells. These are committed to becoming either vascular endothelial cells (endothelioblasts) or lymphangioblasts (committed to becoming lymphatic endothelium). Lymphatic endothelial cells express VEGFR-3. VEGF-C and VEGF-D signal through VEGFR-3 to induce the proliferation, activation and migration of lymphatic endothelial cells (lymphangiogenesis). In the adult, VEGFR-3 has been shown to be expressed only on lymphatic and new vascular (neoangiogenic) endothelial cells. Another specific marker for lymphatic endothelial cells is the podocyte cell-surface mucoprotein podoplanin. The origin of the endothelial tumours is usually confirmed by the expression of blood and lymphatic endothelial-specific markers in neoplastic cells. For example, spindle cells which constitute Kaposi's sarcoma (KS) tumours have always been thought to belong to the endothelial lineage. The fact that KS spindle cells express both VEGFR-3 and podoplanin, but not the capillary/venous PAL-E cell-surface marker (as vascular endothelial cells do), indicates that these cells represent mature lymphatic or lymphatic precursor cells. It is possible that Kaposi's-sarcoma-associated herpesvirus (KSHV) exploits the endothelial-cell developmental process to promote its own replication, similar to the manner in which Epstein–Barr virus (EBV) and human papillomavirus (HPV) exploit normal B cell and epithelial-cell differentiation pathways, respectively. Like other viral-induced cancer cells, it is unlikely that these spindle cells represent terminally differentiated cells. Neoplastic cells in many other endothelial tumours (e.g. angiomas and lymphangiomas) express markers of both lymphatic and blood vessel endothelium. Therefore the origin of the majority of these tumours remains enigmatic. Tumour endothelial cells in non-endothelial tumours (see also Fig. 1) derive from either normal endothelium or from endothelial progenitors that acquire specific properties and express specific markers [e.g. tumour endothelial markers (TEM) 1,5,7 and 8] that are not expressed in normal endothelium. (Adapted from Boshoff and Weiss 2002, and further modified)

endothelial cells isolated from various vascular beds is often organ-specific (Chi et al. 2003). Finally, tumour endothelium is also different from normal endothelium within the same organ (St Croix et al. 2000). To accommodate local



physiological requirements, endothelial cells acquire specialised characteristics and functions that are determined in part by the host tissue (Risau 1998).

The tumour microenvironment in particular has a profound effect on the transcriptome of endothelial cells. Cytokines and angiogenic molecules secreted by cancer and immune cells modulate the expression of cellular adhesion molecules and other surface markers on the tumour endothelium. For example, VEGF and tumour-necrosis factor (TNF)-α up-regulate, whereas transforming growth factor (TGF)-β1 down-regulates, adhesion molecules (Jain et al. 1996). Because the expression and activity of general angiogenic factors such as VEGF and Ang-1 vary greatly in different tumours, these determine EC heterogeneity and the possibility of the organ-specific mechanisms of angiogenesis (LeCouter et al. 2002; St Croix et al. 2000). Tumour vessels change their phenotype and express surface proteins that are absent or barely detectable in quiescent vessels, with the "molecular signature" of tumour endothelium largely depending on the tumour type (Eliceiri and Cheresh 1999; Huang et al. 1997; Lal et al. 2001; St Croix et al. 2000). Epitopes specific for tumour endothelial cells represent attractive targets for anti-angiogenic therapy (Fig. 2; described in more details in Sect. 5).

2.2 Endothelial Progenitor Cells in Tumour Neovascularisation

Gene expression analysis comparing EPCs to endothelial cells has revealed that human endothelial cell precursors resemble freshly isolated endothelial cells from tumours, rather than cultured endothelial cells (Bagley et al. 2003). Recent studies demonstrate that angiogenic factors and chemokines direct EPCs to tumour neovessels (Spring et al. 2005). During tumour angiogenesis (described in more details in Sect. 5) the levels of circulating VEGF have been shown to rise (Connolly et al. 1989a, b; Leung et al. 1989; Senger et al. 1986). Increased circulating VEGF promotes the mobilisation of EPC from the BM, resulting in increased numbers of circulating endothelial progenitors in various pathologies including neoplasms (Asahara et al. 1999; Hattori et al. 2001). Human BM-derived cells have been shown to infiltrate human tumours (Peters et al. 2005) and to give rise to up to 16% of the tumour neovasculature in normal mice, complementing resident endothelial cells in sprouting new vessels (Ruzinova et al. 2003). Peters et al. analysed the tumour endothelial cells in six individuals who developed cancers after bone-marrow transplantation with donor cells derived from individuals of the opposite sex and found that an average of 4.9% of cells of the total endothelial cell population were derived from the BM (Peters et al. 2005). In contrast to these studies, De Palma et al. suggested that the percentage of EPCs that are truly incorporated into a growing vessel wall is very low and that the majority of BM-derived cells homing in on the tumour vasculature are adherent perivascular mononuclear cells, which may contain angiogenic factors (De Palma et al. 2003). Further-

Table 2 Factors affecting endothelial cells differentiation and function during vasculogenesis, angiogenesis and lymphangiogenesis

Factor	Receptor	Function
VEGF family members (VEGF-A, -B, -C, -D; PlGF) (secreted factors)	VEGFR-1, VEGFR-2, VEGFR-3 (receptor tyrosine kinases)	Stimulate angio/vasculogenesis (differentiation of haemangioblasts and proliferation and migration of mature endothelial cells), permeability
VEGF-C; VEGF-D (secreted factors)	VEGFR-3 (receptor tyrosine kinase)	Lymphangiogenic factors (differentiation, growth and remodelling of lymphatic vessels)
Ang-1 (secreted factor)	Tie-2 (receptor tyrosine kinase)	Stabilise vessels, inhibit permeability and EPC release from BM
Ang-2 (secreted factor)	Tie-2 (receptor tyrosine kinase)	Antagonist of Ang-1, destabilises blood vessels; plays role in lymphangiogenesis
	Integrins $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_5\beta_1$ (receptors for matrix macromolecules and proteinases)	Regulate endothelial cells attachment to extracellular matrix molecules, migration and survival, as well as vasculogenesis
bFGF	FGFR	Stimulate angio/arteriogenesis and angioblast differentiation
VE-cadherin; PECAM (CD31) (endothelial junctional molecules)		Stabilisevessels, inhibit permeability
SDF-1 (secreted factor)		Stimulate mobilisation and differentiation of BM-derived progenitor cells, vasculogenesis

Table 2 (continued)

Factor	Receptor	Function
Ephrins (ligands tethered to membrane)	Eph receptors (receptor tyrosine kinases)	Regulate arterial/venous specification during EPC differentiation
TGF-β1, endoglin	TGF-β receptors	Stimulate extracellular matrix production
AC133 (glycoprotein)		Regulate angioblast differentiation
PDGF-A and -C	PDGFR-α and -β	Recruitment of angiogenic stroma
PDGF-B	PDGFR-β	Vessel maturation through recruitment of smooth muscle cells
Plasminogen activators, MMPs		Remodel matrix, release and activate growth factors such as bFGF
EG-VEGF	EG-VEGFR-1 and EG-VEGFR-2 (GPCRs)	Organ-specific angiogenesis
Adrenomedullin	Heterodimeric complex formed by GPCR CL and one of the RAMPs (1,2 or 3)	Stimulate vasculo/angiogenesis (differentiation of EPC; proliferation, migration and network formation of mature endothelial cells) and vasodilatation; regulate permeability
Chemokines	Various	Pleiotropic role in angiogenesis
Prox-1 (homeobox transcription factor)		Growth and differentiation of lymphatic endothelium
	Integrin $\alpha_9\beta_1$(receptor for matrix macromolecules and proteinases)	Lymphangiogenesis

Table 2 (continued)

Factor	Receptor	Function
Foxc2 (transcription factor)		Lymphangiogenesis (lymphatic patterning, negative regulator of lymphatic vessel hyperplasia)
	Soluble VEGFR-1 Soluble NRP-1	Sink for VEGF, VEGF-B, PlGF
Angiostatin and related plasminogen kringles		Suppress tumour angiogenesis
Endostatin (collagen XVIII fragment)		Inhibit endothelial growth
Vasostatin; calreticulin		Inhibit endothelial growth
TIMPs, MMP inhibitors		Suppress pathological angiogenesis

Abbreviations: EPC, endothelial progenitor cells; BM, bone marrow; VEGFR, VEGF receptor; PlGF, placental growth factor; NRP-1, neuropilin-1; MMP, matrix metalloproteinase; TIMPs, tissue inhibitors of MMP; SDF-1, stroma-derived factor; HGF, hepatocyte growth factor; CL, calcitonin-receptor-like receptor; RAMP, receptor activity modifying protein; EG-VEGF, endocrine gland-derived VEGF; GPCR, G protein-coupled receptor; Based on: Carmeliet and Jain (2000); Alitalo et al. (2005); Ferrara and Kerbel (2005); Yancopoulos et al. (2000); additional selected references are in the text

more, EPCs deriving from other tissue sites might also contribute to tumour angiogenesis.

Thus, BM-derived cells appear to contribute to tumour angiogenesis, of which a small and variable proportion is probably true EPCs. The contribution (as well as precise nature) of EPCs to angiogenesis is still an intensively debated issue. However, it is clear that enumeration of circulating EPCs in individuals with cancer may be useful in predicting the outcome of therapy or disease course (Bertolini et al. 2003; Schmidt-Lucke et al. 2005). Studies in mice indicate that the number of circulating EPCs is affected by systematic exposure to angiogenic regulators such as VEGF and can decline in response to anti-angiogenic treatments such as anti-VEGFR-2 antibody therapy (Shaked et al. 2005). Chemotherapeutic drug responses can be measured in part by the level of EPCs in the circulation (Bertolini et al. 2003). Thus, measurement of circulating EPCs may provide a useful assessment of disease susceptibility or response to therapies. However, controversies and investigation concerning the phenotype of circulating EPCs have not completely resolved which circulating cells give rise to endothelial cells during in vivo angiogenesis and lymphangiogenesis in tumours (Oliver 2004).

The observation that VEGFR-1^+ (VEGF receptor 1, described in Sect. 3.1) BM-derived haematopoietic cells provide a niche for tumour metastases (Kaplan et al. 2005) further opens the debate on the relationships between haematopoietic and endothelial precursors and their role in tumour angiogenesis and growth.

3
Angiogenesis and Lymphangiogenesis in Cancer

Vessels can grow in several ways. *Vasculogenesis* refers to the formation of blood vessels by endothelial progenitors (as described in Sect. 2), while *angiogenesis* and *arteriogenesis* refer to the sprouting of pre-existing vessels and subsequent stabilisation of these sprouts by mural cells. Collateral growth denotes the expansive growth of pre-existing vessels, forming collateral bridges between arterial networks (Carmeliet 2003). When vessel growth is deregulated, it has a major impact on our health and contributes to the pathogenesis of many disorders. Excessive angiogenesis or lymphangiogenesis occurs in cancer, psoriasis, arthritis and blindness. In cancer, the blood vessel network is expanded to meet the demand of a growing tumour mass, and lymphatic vasculature provides the path for metastasising cells. Other common disorders associated with abnormal blood vessel growth include obesity, atherosclerosis and inflammatory diseases (Table 1; Fig. 1). In addition, abnormal angiogenesis or lymphangiogenesis and endothelial transformation are hallmarks of infectious diseases and several *endothelial neoplasms* (e.g. Kaposi's sarcoma, haemangioma and lymphangioma; Table 1; Fig. 1).

3.1 Tumour Angiogenesis

Blood vessels in the embryo form through vasculogenesis; that is, through in situ differentiation of undifferentiated precursor cells (angioblasts) into endothelial cells which assemble into a vascular labyrinth (Risau 1997). Subsequently, this primitive network expands by angiogenesis (sprouting or intussusception from pre-existing vessels) (Patan et al. 1996). In the adult, physiological neovascularisation occurs in the female reproductive tract (e.g. during ovulation) and during wound healing. Pathological angiogenesis is a hallmark of cancer and various ischaemic and inflammatory diseases (Table 1). Vascular density often correlates with tumour grade (differentiation) and prognosis in various malignancies (Fox and Harris 2004).

Tumour vessels develop by angiogenesis (sprouting or intussusception from pre-existing vessels) or by co-option of normal vasculature by tumour cells (Carmeliet and Jain 2000). Circulating endothelial precursors, shed from the vessel wall or mobilised from BM, also contribute to tumour angiogenesis (Asahara et al. 2000; Rafii 2000; Fig. 1). Tumour angiogenesis requires the coordinated action of a variety of growth factors and cell-adhesion molecules in tumour, endothelial and mural cells (Coultas et al. 2005; Table 2). Amongst these, members of the VEGF and Ang family play a predominant role (Carmeliet and Jain 2000; Yancopoulos et al. 2000; Table 2).

VEGF-A is central in tumour angiogenesis (Ferrara 2004; Table 2). VEGF-A binds to two receptor tyrosine kinases (RTK), VEGFR-1 (Flt-1) and VEGFR-2 (KDR, Flk-1). Of the two, it is now generally agreed that VEGFR-2 is the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF-A. Mice engineered to lack VEGFR-2 fail to develop a vasculature and have few endothelial cells (Shalaby et al. 1995).

The significance of VEGFR-1 in the regulation of angiogenesis is more complex. Under some circumstances, VEGFR-1 may function as a "decoy" receptor that sequesters VEGF-A and prevents its interaction with VEGFR-2 (Ferrara and Kerbel 2005). However, there is growing evidence that VEGFR-1 has significant roles in haematopoiesis and in the recruitment of monocytes and other BM-derived cells that home in on the tumour vasculature and promote angiogenesis (Gerber et al. 2002; Hattori et al. 2002; Luttun et al. 2002). During sprouting angiogenesis, vessels initially dilate and become leaky in response to VEGF-A. Members of the angiopoietin family, such as Ang-1 and Ang-2, are required for further remodelling and maturation of the vasculature through recruitment of mural cells (Yancopoulos et al. 2000). Ang-2 might also play a direct role in tumour angiogenesis and lymphangiogenesis (Alitalo et al. 2005; Oliner et al. 2004). A number of other activators, as well as inhibitors, of tumour angiogenesis have been identified and these molecules have an established role in the development and differentiation of the vessel wall (Table 2). Thus, platelet-derived growth factor (PDGF)-B is required for recruitment of

pericytes and maturation of the microvasculature (Lindahl et al. 1997). Furthermore, recent studies have emphasised the significance of tumour-derived PDGF-A (and potentially PDGF-C) and PDGFR-α signalling in the recruitment of an angiogenic stroma (heterogeneous compartment comprising fibroblastic, inflammatory and immune cells) which produces VEGF-A and other angiogenic factors (Dong et al. 2004). Finally, negative regulators of angiogenesis, including thrombospondin, vasohibin and several fragments of larger proteins including angiostatin, tumstatin and vasostatin have been identified (Table 2). The precise role of these proteins during tumour angiogenesis remains to be clearly defined, although several hypotheses have been proposed, including that they bind to specific integrins and affect endothelial cell migration and survival in the case of endostatin and tumstatin (Ferrara and Kerbel 2005).

Maintenance of new vessels depends largely on the survival of endothelial cells. Endothelial apoptosis in neovasculature is induced through deprivation of nutrients or survival signals (Carmeliet and Collen 1999; Gerber et al. 1999; Jain et al. 1998). VEGF [through its interaction with vascular endothelial (VE)-cadherin] (Carmeliet and Collen 1999) and Ang-1 are vital survival factors for tumour neovasculature. Their depletion causes tumour vessels to regress, especially when vessels have only been recently assembled and are still immature. This can occur as a result of insufficient maturation of newly formed vessels because cross-talk between endothelial and mural cells is essential in maintenance of a functional vasculature. In contrast, most angiogenesis inhibitors cause endothelial apoptosis directly through interaction with cell-surface molecules in endothelial cells (Table 2). Endothelial apoptosis can also be induced by nitric oxide, reactive oxygen species, interferon-γ and VEGF pathway inhibitors (described in Sect. 5).

Hypoxia is a strong stimulus for angiogenesis in numerous disorders including cancer (Harris 2002). Hypoxia is a frequent feature of the microenvironment of solid tumours and constitutes one of the driving forces of cancer growth and progression. Cells in tumours become hypoxic when too distant from nearby vessels. Hypoxia activates hypoxia-inducible transcription factors (HIFs), which function as master switches to induce the expression of several angiogenic factors, including VEGF, nitric oxide synthase (NOS), placenta-derived growth factor (PlGF), Ang-2, adrenomedullin and others produced by tumour and inflammatory cells (Table 2; Harris 2002). HIF operates in concert with the product of the von Hippel–Lindau (VHL) tumour suppressor gene. Under normoxic conditions, the VHL protein targets HIF for ubiquitination and degradation (Safran and Kaelin 2003). Inactivating VHL mutations occur in about 50% of renal cell carcinomas, where particularly high levels of VEGF-A expression have been found (Seizinger et al. 1988).

The up-regulation of VEGF-A and other angiogenic factors (Table 2) is not only linked to hypoxia or VHL mutations. A very broad and diverse spectrum of oncogenes is associated with up-regulation of angiogenic factors, including mutant ras, erbB-2/Her2, activated EGFR and bcl-abl in tumour cells (Ker-

bel et al. 2002; Rak et al. 1995). Viral oncogenes are also implicated in the up-regulation of members of VEGF and Ang families of angiogenic factors in endothelial cells in Kaposi's sarcoma (described in Sect. 4.4) (Wang et al. 2004). Besides VHL, inactivation/mutation of various other suppressor genes also result in the upregulation of VEGF expression in tumour and stroma cells (Brugarolas and Kaelin 2004). In other endothelial cell neoplasms, such as haemangioma and lymphangioma, pathological angiogenesis relies on expression of known growth factors, but the mechanisms regulating their up-regulation remain unknown (Ritter et al. 2002; described in Sect. 5).

3.2 Lymphangiogenesis and Cancer Progression

The lymphatic vasculature forms a vessel network that drains extravasated interstitial fluid proteins and cells from tissues and returns them back to the venous blood circulation. Lymphatic vessels are also an essential part of the body's immune defence. Deregulated lymphangiogenesis has an important role in the pathogenesis of several diseases such as lymphoedema, various inflammatory conditions and cancer. Malignant tumours expressing lymphangiogenic factors can directly activate lymphangiogenesis and lymphatic metastasis (Karpanen et al. 2001; Mandriota et al. 2001; Skobe et al. 2001; Stacker et al. 2001).

3.2.1 Differentiation of Lymphatic Endothelium in the Embryo

Lymphatic endothelial cells arise by sprouting from embryonic veins in the jugular and perimesonephric areas (Sabin 1909). From here they migrate to form primary lymph sacs and the primary lymphatic plexus, which is composed of capillary-like vessels (Alitalo et al. 2005). The homeobox transcription factor Prox1 is essential for these initial developmental events. The immature lymphatic endothelial cells (LECs) that are in a process of terminal differentiation express specific markers such as LYVE-1 (lymphatic vessel hyaluronan receptor-1). As differentiation and lymphangiogenesis progress, additional lymphatic markers are expressed during formation of lymph vessels and capillaries (Fig. 2).

Studies over the past 10 years revealed a signal transduction system for LEC differentiation, growth, migration and survival. This system is formed by VEGF-C and VEGF-D and their receptor VEGFR-3 (Achen et al. 1998; Joukov et al. 1996; Kaipainen et al. 1995; Makinen et al. 2001). These molecules play a significant role in lymphangiogenesis (Oliver 2004). The discoveries of specific markers of lymphatic endothelium and key lymphangiogenic factors have enabled the study of the lymphatic vasculature in tumours.

3.2.2
Molecular Regulation of Lymphangiogenesis in Cancer

Tumour cells activate peri-tumoural and intra-tumoural lymphangiogenesis as demonstrated by the use of lymphatic-specific molecular markers (Beasley et al. 2002; Karpanen et al. 2001; Mandriota et al. 2001; Skobe et al. 2001; Stacker et al. 2001; Wigle et al. 2002; Fig. 1). Proliferating intra-tumoural lymphatic vessels are present in certain human cancers, such as melanomas, head and neck carcinomas and xenograft tumour models overexpressing lymphangiogenic factors (Dadras et al. 2003; Maula et al. 2003). VEGF-C- and VEGF-D-induced lymphangiogenesis mediates tumour cell dissemination (metastasis). In mouse models, the induction of tumour lymphangiogenesis by VEGF-C promotes breast cancer metastasis (Skobe et al. 2001). Levels of VEGF-C expression by some types of primary tumours seem to correlate with the degree of lymph-node metastasis (Karpanen et al. 2001; Mandriota et al. 2001). VEGF-D also induces the formation of intra-tumoural lymphatic vessels in a mouse tumour model, and its expression by tumour cells facilitates the spreading of the tumour to regional lymph nodes (Stacker et al. 2001). A direct link between VEGF-C or VEGF-D expression and metastasis was established with the use of a soluble VEGFR-3-immunoglobulin fusion protein (VEGF-C/D trap) or blocking anti-VEGF-D antibodies (He et al. 2002; Karpanen et al. 2001; Stacker et al. 2001).

Although these studies provide support for the contribution of VEGF-C, VEGF-D and their receptor, VEGFR-3, in lymphatic metastasis, the mechanisms of these effects and the manner by which tumour cells enter the lymphatic system have only recently been addressed. For example, some evidence indicates that the lymphatic endothelium actively participates in metastasis formation by secreting chemokines such as CCL21 (SLC, 6Ckine and Exodus), whose receptor (CCR7) is expressed on some tumour cells (Zlotnik 2004). Finally, the presence of functional lymphatics around tumours appears to be sufficient for lymphatic metastasis, suggesting that intra-tumoural lymphatics are not always required (Padera et al. 2002; Fig. 1).

VEGFR-3 might also play a role in the infection of endothelial cells by KSHV (or human herpesvirus-8, HHV8) (Zhang et al. 2005) which then leads to transformation and reprogramming of the endothelium (Hong et al. 2004; Wang et al. 2004; described in Sect. 4.4). KSHV envelope glycoprotein gB interacts with VEGFR-3 and $\alpha_3\beta_1$ integrin and activates both, resulting in endothelial cell growth and migration (Zhang et al. 2005). VEGF-C and VEGFR-3 are co-expressed in endothelial cells in lymphangiomas (neoplasms of lymphatic vessels; described in Sect. 4), suggesting that these molecules may take an active part in the formation of these endothelial neoplasms by autocrine or paracrine regulation (Huang et al. 2001).

Although previous and current studies enabled progress towards understanding the biology of the lymphatic system, important questions remain unanswered. Are new lymphatic vessels and capillaries formed only by the

proliferation of endothelial cells from pre-existing lymphatics, or can they also be formed from endothelial precursors, lymphangioblast cells or by endothelial budding from veins? One study has shown that lymphatic endothelial progenitor cells derived from the circulation contribute to inflammation-associated lymphangiogenesis in human renal transplants, but not to tumour lymphangiogenesis (Kerjaschki et al. 2006).

4 Neoplasms of Endothelial Cells

Unrestricted proliferation of endothelial cells contributes not only to the development of non-endothelial tumours (e.g. epithelial cancers) but also to the pathogenesis of several endothelial neoplasms (Fig. 1; Table 1). These "*endothelial tumours*" are heterogeneous in their clinical description and behaviour, as well as in their aetiology. They are mainly formed of blood or lymph vessels (i.e. endothelium), with supporting tissues, and represent developmental defects or true neoplasms.

Haemangiomas are benign tumours of the vascular endothelium and are the most common tumours of infancy (Bell 2003; Ritter et al. 2002). These neoplasms are incapacitating, but little is known about their aetiology (Vikkula et al. 1998). Haemangiomas are characterised by an initial phase of rapid proliferation, which is followed, in most cases, by spontaneous involution. Although most lesions resolve without complication, there are circumstances in which these tumours become vision- or life-threatening, e.g. occurring near the eye, airway or other vital structures. VEGF and bFGF are implicated in their progression (Bielenberg et al. 1999). Interferon-β was identified as a potential endogenous inhibitor of this tumour, because it was found to be expressed in the epidermis underlying involuted, but not proliferating, haemangiomas. Therefore it was suggested that an imbalance in positive and negative regulators of angiogenesis is associated with the development of this tumour. Although these studies have described biological characteristics of haemangiomas in different phases and identified pathways which could be involved, the causative factors in haemangioma growth and involution remain to be identified (Ritter et al. 2002).

Lymphangiomas are benign neoplasms of lymphatic vessels. Derailed growth of VEGFR-3/podoplanin-positive lymphatic vessels results in lymphangioma, with subsequent formation of secondary lymphoedema due to impaired lymph fluid drainage. Lymphangiomas affect most organs, although they are most commonly found in the soft tissues of the head, neck and axilla, where they consist of a benign multi-cystic mass of dilated lymphatic channel networks (Alitalo and Carmeliet 2002).

Angiosarcomas and *lymphangiosarcomas* are malignant endothelial tumours of blood and lymphatic vessels respectively. In these malignancies, the neoplastic endothelial cells autonomously produce (lymph)angiogenic growth factors

and VEGF, as well as angiopoietins and Tie2 receptors (Brown et al. 2000; Zietz et al. 1998). Increased expression of the p53 tumour suppressor and mdm-2 proto-oncogene proteins leads to a loss of regulation of VEGF expression through decreased thrombospondin-1 regulation in angiosarcomas (Zietz et al. 1998).

Recent studies provide insight into the transcriptional profile and possibly an origin of some of these endothelial tumours (Hong et al. 2004; Ritter et al. 2002; Wang et al. 2004). These neoplasms may arise from genetic alterations or viral infections. For instance, products of KSHV and human immunodeficiency virus type-1 (HIV-1) have been implicated in the pathogenesis of Kaposi's sarcoma, found in approximately 30% of AIDS patients (Albini et al. 1996; Boshoff et al. 1995; Chang et al. 1996; Flore et al. 1998). HIV-1 Tat protein activates VEGFR-2, binds endothelial $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins and retrieves bFGF from the extracellular matrix (Albini et al. 1996). In general, Kaposi's sarcoma remains the most studied amongst endothelial tumours, and recent studies provide further insight into the pathogenesis of this neoplasia.

Kaposi's sarcoma is a neoplasm characterised by vascular nodules in the skin, mucous membranes and internal organs. It is endemic in sub-Saharan regions in Africa and is frequently encountered in HIV-1-infected individuals (Boshoff and Weiss 2002). The nodules are composed of clusters of spindle-shaped tumour cells and characterised by a prominent vasculature. The finding that spindle cells and cells lining the irregular vascular spaces in Kaposi's sarcoma express both blood and lymphatic endothelial cell markers suggests their endothelial origin (Fig. 3). Development of Kaposi's sarcoma is associated with infection by KSHV (Chang et al. 1994). The transcriptional profile of Kaposi's sarcoma is akin to LECs (Hong et al. 2004; Wang et al. 2004; Fig. 3). Furthermore, in vitro infection of blood vascular endothelial cells with KSHV resulted in the expression of several lymphatic endothelial cell-specific genes, although KSHV-infected lymphatic endothelial cells also showed some infidelity of phenotypic gene expression (Hong et al. 2004; Wang et al. 2004). It is still unclear whether KSHV primarily infects LECs to precipitate Kaposi's sarcoma, or whether the virus infects EPCs and steers their differentiation towards a LEC genotype.

KSHV encodes for a number of proteins that could directly play a role in endothelial proliferation. These included a viral cyclin (Chang et al. 1996), a viral FLICE inhibitor (vFLIP), and a latency-associated nuclear antigen (LANA). LANA interacts with both p53 and pRb, and can transform rodent cells (Radkov et al. 2000). KSHV also encodes for a number of cellular homologues, which could play a direct role in promoting angiogenesis. These include a viral encoded G protein-coupled receptor (vGPCR) (Bais et al. 2003), and a number of chemokine homologues such as macrophage inflammatory proteins (vMIPs) (Boshoff et al. 1997).

Most spindle cells in Kaposi's sarcoma lesions only express KSHV latent genes (including v-cyclin, vFLIP and LANA), but a fraction of cells also ex-

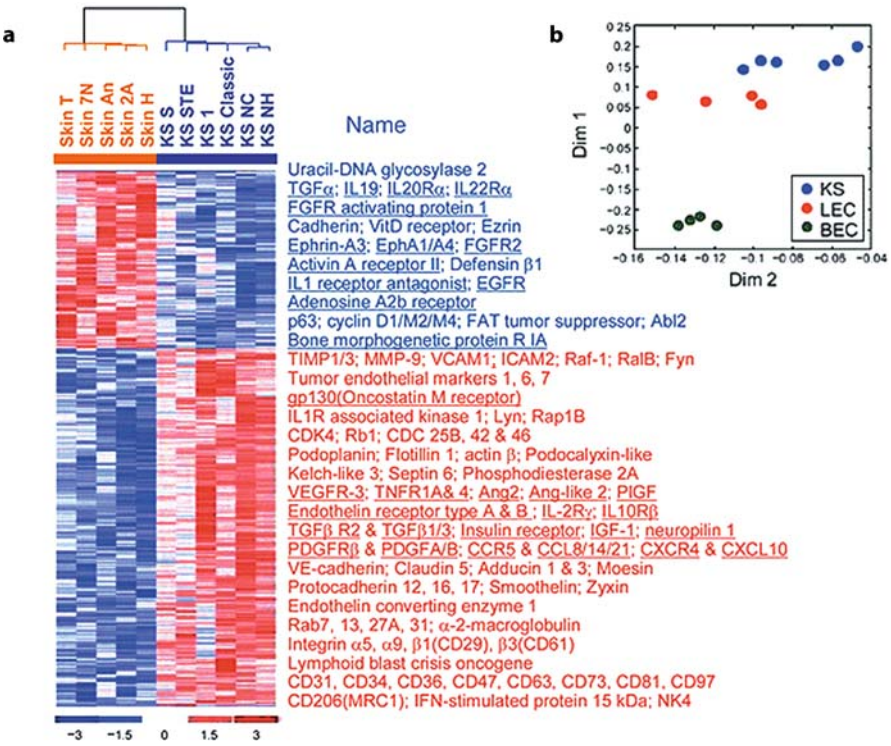


Fig. 3 a,b Relationship between the expression signature of Kaposi's sarcoma and blood and lymphatic endothelial cells. **a** Gene expression microarray (GEM) data for Kaposi's sarcoma is shown through a heat map of 1,482 genes that differentiate sarcoma (KS) and skin sample groups ($p \leq 0.05$). Selected down-regulated genes are shown in *blue* and upregulated genes in *red*. Cytokines and chemokines and their receptors are *underlined*. *Colour scale* indicates units of standard deviation (SD) from the mean expression of each gene. **b** Multi-dimensional scaling (MDS) plot using the lymphatic and blood vessel endothelial cell (LEC-BEC) discriminatory gene signature $n=114$; $p \leq 0.2$). (Adapted from Wang et al. 2004)

press lytic proteins. These lytic proteins (including vGPCR and vMIPs) might attract EPCs to lesions, where they are infected by the virus. Latent and lytic infection therefore appears to be important in the maintenance of this endothelial tumour.

5 Targeting Endothelium

Inhibiting angiogenesis and lymphangiogenesis is a promising strategy for the treatment of cancer. Major progress has been achieved over the past few years, and there is now proof that an anti-angiogenic approach, when combined

with chemotherapy, results in increased survival in patients with advanced malignancies (Ferrara and Kerbel 2005). The majority of these approaches are focussed on targeting tumour endothelium and mechanisms regulating its survival, proliferation and migration, as well as inhibition of EPC recruitment to sites of neovascularisation in tumours.

Many angiogenesis inhibitors are currently in phase I, II or III clinical trials. The inhibitors tested include agents with diverse mechanisms of action (several of which are not known). At present, inhibitors of the VEGF pathway are the most clinically advanced. Several strategies exist to inhibit VEGF signalling. These include monoclonal antibodies targeting VEGF-A (e.g. bevacizumab) or VEGF receptors. Bevacizumab, a humanised variant of a murine anti-VEGF-A monoclonal antibody used in early proof-of-concept studies (Kim et al. 1993), is the only current FDA-approved specific anti-angiogenic treatment for cancer (Ferrara 2004). Chimaeric soluble receptors such as "VEGF-trap" (domain 2 of VEGFR-1 and domain 3 of VEGFR-2 fused to the Fc fragment of an antibody) are also undergoing clinical development. Additional extracellular inhibitors are aptamers that bind to the heparin-binding domain of VEGF165 (pegaptanib). A variety of small-molecule VEGF RTK inhibitors that inhibit ligand-dependent receptor autophosphorylation of VEGFR-1 and VEGFR-2 are being tested. Additional strategies to inhibit VEGF signalling include antisense and siRNA targeting VEGF-A and its receptors.

Cell surface molecules that are preferentially expressed in tumour, but not normal, endothelium are attractive targets for anti-angiogenic therapies. The tumour vascular bed-specific expression of a variety of proteins, including cell-surface antigens, has been evaluated (Aird et al. 1997; Arap et al. 1998; St Croix et al. 2000). In vivo selection of phage display libraries has yielded peptides (for example, amino acid sequences RGD and NGR) that preferentially recognise vessels in subcutaneous tumours in mice (Arap et al. 1998). These peptides can be used to target therapeutic agents to tumours. The serial analysis of gene expression (SAGE) identified molecules preferentially expressed on endothelium in blood vessels in malignant colorectal tissues (St Croix et al. 2000). These molecules are conserved tumour endothelial markers in mice and human and therefore could also present attractive targets for the development of anti-angiogenic therapies (Carson-Walter et al. 2001). The challenge now is to discern how specific these "vascular zip codes" are, as targeting drugs to the tumour vasculature has the potential to change the paradigm for cancer treatment.

The recent discovery of lymphangiogenic factors VEGF-C and VEGF-D and their receptor VEGFR-3 (see Sect. 3.2.1) has opened novel diagnostic and therapeutic avenues for anti-lymphangiogenic therapy, and for the treatment of lymphoedema and metastasis in particular. Blocking monoclonal antibodies that target these factors or their receptor(s) and small molecules that inhibit the tyrosine kinase catalytic domain of these receptors could be used for the inhibition of tumour metastasis. For example, lymphatic spreads induced by VEGF-D

were blocked with an antibody specific for VEGF-D (Stacker et al. 2001). Also, VEGF-C-induced tumour growth, lymphangiogenesis and intralymphatic tumour growth were inhibited by adenoviral expression of the soluble VEGFR-3 receptor, which "traps" available VEGF-C and VEGF-D (Karpanen et al. 2001). However, caution is warranted, since destruction of lymphatic vessels could further elevate the already increased interstitial fluid pressure inside the tumours, thereby further impairing the delivery of other anti-cancer drugs.

Inhibition of EPC mobilisation from the BM also has tremendous therapeutic potential, as evidenced by the inability of tumours to grow in animals that lack functional EPCs (Lyden et al. 1999, 2001). Preliminary work in animal models suggests that agents that inhibit EPC mobilisation may comprise an effective cancer therapy (Capillo et al. 2003), but further work is needed before this strategy can be applied to human neoplasms. This includes endothelial neoplasms such as Kaposi's sarcoma, where the contribution of KSHV-infected EPCs as well as resident differentiated endothelium has been suggested (Pyakurel et al. 2006).

As the cellular and molecular mechanisms of angiogenesis differ in various tissues (Carmeliet 2000, 2003), the therapeutic inhibition of angiogenesis should be adjusted to the target tissue. The recent discovery of tissue-/organ-specific regulators of angiogenesis, such as endocrine gland-derived VEGF, suggests a potentially novel approach to this problem (LeCouter et al. 2002). A principal benefit of tissue-specific angiogenic therapeutics could be the elimination of systemic, undesired effects associated with broad-spectrum angiogenic molecules. Furthermore, in endothelial neoplasms, where mechanisms of angiogenesis are not well-characterised, alternative therapies could be considered, depending on the outcome of studies investigating the cellular and molecular mechanisms involved.

6 Conclusions

Both the blood and lymphatic endothelial systems are central in the pathogenesis of human malignancies. Lymphatics are often the first port of call for tumour metastases, and blood vessels provide essential nutrients and oxygen supply to promote tumour cell proliferation. The secretion of pro-angiogenic and lymphangiogenic molecules by tumour cells initiates the growth of these vessels. Recent evidence also indicates that circulating endothelial cells and endothelial precursor cells are mobilised by the growing tumour mass, further contributing to angiogenesis and the cytokine environment enabling tumour cell proliferation. Understanding the molecular mechanisms underlying the proliferation of blood and lymphatic endothelial cells should lead to novel ways to prevent and treat cancer.

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