Review

Angiogenesis and the role of bone marrow endothelial cells in haematological malignancies

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Summary. Increased microvessel density (MVD) has been observed in the bone marrow (BM) of patients with multiple myeloma (MM), acute lymphoblastic leukaemia, acute myeloid leukaemia, and myelodysplastic and myeloproliferative syndrome. The MVD is the net result of cumulative phases of angiogenesis and angio-regression and is as such not an indicator of the ongoing angiogenesis at the time of biopsy. There is, therefore, a need for additional methods that allow the estimation of ongoing angiogenesis. Double immunostainings for CD34 and Ki-67 can be used on paraffin-embedded tissue to determine the endothelial proliferation fraction. The BM endothelial cells, as a component of the BM stroma, have a close interaction with the malignant cells. In MM, for example, they are involved in the specific homing and are a source of paracrine growth factors. Targeting the BM microvessels will not only influence the nutrient and oxygen supply, but will in addition reduce the growth stimuli provided by the EC.

Key words: Haematological malignancies, Angiogenesis, Hypoxia, Endothelial cell, Microvessel density, Endothelial cell proliferation

Introduction

More than a century ago the surgeon Stephen Paget (1898) suggested that the site of metastasis depended on the affinity of the tumour for the micro-environment with his seed and soil hypothesis. Currently, it is evident that for the study of the pathophysiology of tumour growth all components of the microenvironment should be considered (Compagni and Christophori, 2000; Bissell and Radisky, 2001; Almholt and Johnsen, 2003; Christofori, 2003). For haematological malignancies there is clear evidence of an intimate interaction between

tumour cells and stromal cells (Hideshima and Anderson, 2002). The microvasculature is an active component of the stroma. This vasculature is responsible for the appropriate oxygen and nutrient supply and the removal of waste products. It provides a route for homing and metastasis, and, last but not least, the bone marrow endothelial cells (BMEC) are also involved in autocrine interactions and paracrine interactions with tumour cells. For instance, BMEC secrete vascular endothelial growth factor (VEGF), basic fibroblastic growth factor (bFGF), matrix metalloproteinase-2 (MMP-2), MMP-9, monocyte chemoattractant protein-1 (MCP-1), which are growth factors, and invasive factors for themselves and for multiple myeloma (MM) cells. On the other hand, VEGF secreted by the MM cells can induce secretion of stem-cell factor (SCF), Flt-3 ligand, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6 and IL-7 (Yamaguchi et al., 1996; Fiedler et al., 1997; Bellamy et al., 1999; Bertolini et al., 2000; Dankbar et al., 2000) by the BMEC which will again stimulate the MM cells.

The process of new vessel formation from pre-existing vessels is called "angiogenesis". The regulation of angiogenesis is dependent on several angiogenic pathways. The unravelling of these pathways opens the possibilities for the development of new therapeutic agents. The balance in secretion of angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2) and of VEGF is important in this process. Ang-1 is the major physiological ligand for the Tie-2. Vessel integrity is preserved in mature vessels by activation by Ang-1 of Tie-2 present on the EC (Maisonpierre et al., 1997). Ang-2 competes with Ang-1 for the binding to the Tie-2 which leads to loosening of vessel structures and dissociation of pericytes (Holash et al., 1999), which makes the EC highly responsive to VEGF.

TGF-ß1 is both pro- and anti-angiogenic: the TGF-ß1-ALK1 pathway induces EC migration and proliferation, whereas the TGF-ß1-ALK5 pathway is a positive regulator of vessel maturation. The balance between both pathways is orchestrated by endoglin, a TGF-ß-binding protein (Goumans et al., 2002).

The activators and inhibitors of angiogenesis act at a local level and serum and/or plasma levels will not always reflect the situation in the compartment of interest. The circulating levels are not only influenced by the amount produced, but are also dependent on their half-life. Moreover, angiogenic factors are also produced by cells other than tumour cells, such as stromal cells, EC, osteoclasts, macrophages, mast cells (Ribatti et al., 2001), immune cells, platelets and MKC (Chou et al., 2002). MMP-9, for example, is secreted by neutrophils (Takafuji et al., 2003), macrophages and mast cells (Kanbe et al., 1999). MMP themselves can liberate angiogenic factors from the extracellular matrix or from the basement membrane. Circulating VEGF is produced by tumour cells, but it can also be released by granulocytes, monocytes, mast cells, thrombocytes (Salgado et al., 2000) and megakaryocytes (Benoy et al., 2002). Mast cell counts are higher in patients with active MM than in non-active MM and MGUS (Vacca et al., 2001). The number of mast cells correlates with BM angiogenesis in B-chronic lymphocytic leukaemia (CLL) (Ribatti et al., 2003a) and in myelodysplastic syndrome (MDS). In the latter both MVD and mast cell counts correlate with tumour progression (Ribatti et al., 2002).

It is clear that angiogenic stimulation is dependent on several factors (Orpana and Salven, 2002) and it is therefore unlikely that the measurement of a single angiogenic factor reflects the entire angiogenic process. It is more likely that every disease has a peculiar angiogenic profile (Di Raimondo, 2003).

Hypoxia as a driving force for angiogenesis

Once a solid tumour exceeds a volume of more than 2 mm^3 the simple diffusion of oxygen and nutrients will be inadequate to fulfil the metabolic needs. For further expansion the tumour can rely on several mechanisms: 1) use of the pre-existing vasculature (co-option) (Holash et al., 1999); 2) development of new vessels from pre-existing vessels (angiogenesis); therefore, BM-derived EC precursors can or cannot be recruited (postnatal vasculogenesis) (de Bont et al., 2001a; Pelosi et al., 2002); 3) tumour cells can mimic the activities of EC and participate in the formation of new vessels (mosaicism) (Chang et al., 2000); and 4) fluid-conducting, matrix-rich meshworks (vasculogenic mimicry) (Hendrix et al., 2003) can develop between the tumour cells.

It is the type of tumour, the growth pattern and the microenvironment which will determine the mechanisms to be used. For instance, in one of the three growth patterns observed in liver metastases of colorectal adenocarcinoma, the hepatocytes of the liver plates are replaced by tumour cells with total conservation of the supportive blood vessels. In this so-called replacement-type of growth pattern the nutritional supply is entirely provided by the pre-existing sinusoids (co-option). The liver metastases with a desmoplastic growth pattern are highly angiogenic (Vermeulen et al., 2001). Angiogenic-independent growth has also been observed in lung tumours (Pezzella et al., 1997).

Hypoxia in the tumour is one of the main driving forces of angiogenesis. Two molecular pathways are involved in this hypoxia-driven angiogenesis: the hypoxia-inducible factor (HIF) pathway and the NFκB pathway. Both pathways may interact to provide (under physiological conditions) tight control of angiogenesis (Royds et al., 1998).

HIF pathway

HIF is a key transcriptional regulator of angiogenic growth factors via an oxygen-sensing process (Semenza, 1999). HIF is also a key regulator of a broad range of cellular and systemic responses to hypoxia (Harris, 2002; Semenza, 2003): genes involved in glycolysis, glucose uptake, metabolism, pH, neurotransmitters, stress-response pathways, cell adhesion, extracellular matrix, cytoskeleton and proteases, oxygen and iron metabolism. One of the genes activated by HIF is the gene for carbonic anhydrase isoenzyme 9 (CA IX). This transmembrane protein is involved in the acid-base homeostasis and counteracts the intracellular acidity accumulating under hypoxic conditions (Wykoff et al., 2000). This CA IX expression has been used as a surrogate marker of tumour hypoxia (Stewart et al., 2002; Swinson et al., 2003). Carbonic anhydrase isoenzyme 2 (CA II), which is also involved in the generation of a pH gradient between extracellular and intracellular compartments, has been shown to be expressed in most patients with myeloid as well as lymphoid leukemic blast cells (Leppilampi et al., 2002).

HIF-1 is a αß-heterodimer. There are several isoforms of both subunits. HIF-1ß is constitutively expressed in the nucleus, whereas the HIF-1α subunit is under the influence of hypoxia; HIF-1α and HIF-2α are closely related and both interact with the hormone-responsive elements to induce transcriptional activity. In an oxygen-rich environment two prolyl residues of HIF-1α are hydroxylated by iron-dependent oxygenases. The hydroxylated HIF will be targeted to the von Hippel-Lindau (VHL) E2 ubiquitin ligase complex resulting in proteasomal destruction of HIF-1α. In a hypoxic state HIF-1α is not degraded and can form a heterodimer with HIF-1ß resulting in the transcription of several genes (Wang et al., 1995) (cfr. supra). Qian et al. (2001) detected high levels of HIF-1α mRNA in BM stromal cells after prolonged exposure of the BM to hypoxia.

The HIF system can also be induced or amplified by oncogenic pathways (insulin-like growth factor-1, epidermal growth factor, mutant Ras and v-scr kinase pathways) (Jiang et al., 1997; Pal et al., 2001) and supressor mutations (PTEN, p53, p14[ARF], pVHL) (Maxwell et al., 1999, 2001; Zundel et al., 2000; Ravi et al., 2000; Fatyol and Szalay, 2001; Wiesener et al., 2001). The mechanisms of oncogenic and growth factor stimulation can act at the level of transcription, translation, stabilisation and activation of the HIF-1α

protein. MM cells also constitutively express HIF-1 protein under normoxic conditions (Feinman et al., 2003). Chronic myeloid leukaemia-(CML) associated onco-protein bcr/abl induces HIF-1α and VEGF gene expression (Mayerhofer et al., 2002). Hypoxia further increases HIF-1α protein levels in several MM cell lines as well as HIF-1-dependent transcriptional activity in ARP-1 cells. Treatment of MM cells with IGF-1 and IL-6, induced HIF-1 DNA binding and HIF-1α protein levels in several MM cell lines. The induction of HIF-1 by IGF-1 and IL-6 is via the AKT/PI3-K and MAP kinase pathways.

HIF is involved in a multitude of pathways in which the components can undergo mutations and clonal selection (Semenza, 2000). Clonal selection of one of the components will inevitably result in co-selection of other components linked to that pathway. This process of clonal selection results in a heterogeneous tumour cell population with a heterogeneously distributed HIF overexpression. This uncoordinated HIF overexpression in space and time will result in an inefficient and disorganised angiogenesis, which contrasts to the co-ordinated physiological angiogenesis observed in processes like wound healing.

Studies with solid tumours have provided evidence indicating that HIF-1 mediates resistance to chemotherapy and radiation (Aebersold et al., 2001; Unruh et al., 2003). Inhibitors of HIF-1 could therefore represent an important new class of therapeutic agent (Semenza, 2003).

NFκ–B

NFκ-B is the second hypoxia-responsive pathway (Royds et al., 1998). Most of the angiogenesis-related genes (VEGF, bFGF, TNFα) have a NFκ-B binding site in or near their promoters. NFκ-B can rapidly transduce hypoxic signals. NFκ-B is a dimer that is inactivated by the formation of a trimer with IκB. This inactive cytoplasmically-located trimer can, under the influence of stimulatory signals, undergo phosphorylation, poly-ubiquination and proteasomal degradation of IκB, thereby liberating the NFk-B dimer. This dimer will be translocated to the nucleus and bind DNA. Alternatively, the inhibitory activity of IκB can be abolished by tyrosine phosphorylation, a process independent of proteasomal degradation. This tyrosine phosphorylation of IκB is the ultimate step of a hypoxia-response cascade for the NFκ-B activation: scr activation, occurring within 15 minutes of cellular exposure to hypoxia, leads to ras and raf-1 kinase activation and ultimately to tyrosine phosphorylation of IκB with subsequent NFκ-B activation. Genes regulated by NFκB are those for the cell adhesion molecules VCAM-1, ICAM-1 and E selectin, the cytokine/growth factors IL-2, IL-6, IL-8, G-CSF and the proto-oncogene c-myc, matrix proteins, matrix-degrading enzymes and genes modulating apoptosis such as p53 (Royds et al., 1998).

In response to environmental non-genotoxic stress such as hypoxia, TNFα is produced by macrophages, binds to its receptor and induces apoptosis. TNFα activates NFκ-B by the raf-1 pathway and promotes binding of NFκ-B to the promoter of p53. p53 has an anti-angiogenic effect by inducting thrombospondin-1 and inhibiting VEGF. Mutations in p53 are widespread. Mutant p53 confers a growth advantage for cells under hypoxic conditions. p53 will accumulate in the nucleus under low oxygen tension. Due to hypoxia, tumour cells with a reduced apoptotic potential (those with a mutation of p53) undergo positive selection. Tumour cells with mutant p53 are able to sustain a longer period of cellular proliferation in hypoxic conditions. Finally the p53-mutated cells will become the dominant clone. Those tumour cells able to survive at low oxygen tension are also more resistant to radio- and chemotherapy. Tumours with mutant p53 elicit a stronger angiogenic response since the mutant p53 has lost its ability to up-regulate the anti-angiogenic agent thrombospondin-1 (Dameron et al., 1994) and as a consequence, the transcriptional activation of the VEGF gene is not blocked anymore (Mukhopadhyay et al., 1995). It has furthermore been shown that p53 targets HIF-1α to degradation via mdm-2. Conversely, upon loss of p53, HIF-1α is no longer degraded, thus supporting tumour angiogenesis together with tumour growth (Ravi et al., 2000).

The tumour-associated vessels are different from normal microvessels

Normal vessels are composed of a monolayer of firmly attached EC in close contact with the vascular basement membrane and surrounded by pericytes (smooth-muscle cells). During physiological angiogenesis the vessels quickly reach full maturation and become stable (quiescent). This process is tightly regulated by the balance between angiogenic factors such as VEGF, bFGF, placental growth factor (PlGF), PDGF, Ang-1, Ang-2 and Tie-2 (tyrosine kinase with Ig and EGF homology-2) (Jain and Munn, 2000). In normal vasculature pericyte association reduces EC proliferation and decreases their dependence on VEGF. In tumours the balance between pro- and anti-angiogenic signals is less tightly regulated (Jain, 2003) and the tumour-associated vessels never reach a quiescent stage. In tumour vessels the pericytes are decreased and are more loosely associated with the vascular basement membrane due to the action of Ang-2 on the Tie-2 receptor (Jones et al., 2001; Vajkoczy, 2002). The EC of tumour vessels have a reduced level of adhesion molecules resulting in a loosening of the cell-cell adhesion and of the adhesion to the extracellular matrix (Dejana, 1996). The EC of tumour vessels are abnormal in shape: they have an increased permeability due to widening of intercellular junctions, a discontinuous basement membrane, transcellular holes, vesicles and fenestrae (Hashizume et al., 2000). They are irregular and tortuous resulting in a turbulent, oscillating blood flow (Mollica et al., 2003) and may become hypoxic because of aberrant oxygen

supply. Tumour cells can be incorporated into the blood vessel lining (Chang et al., 2000).

Such disturbed architecture of the microvasculature has been observed in solid tumours (Jain, 2003) as well as in haematological malignancies. Lundberg et al. (2000) studied the microvessel architecture by the use of serial sections and confocal microscopy: the microvessels in polycythaemia vera (PV) were relatively straight, but more branched than vessels in the BM of normal patients; in chronic myelocytic leukaemia (CML) the BM microvessels were relatively straight with numerous branches; and in idiopathic myelofibrosis (IMF) the microvessels were arranged in vascular nests with numerous short vessels that were highly branched and tortuous. In the blastic transformation of CML the calibre of the microvessels was smaller compared to the chronic phase (Korkolopoulou et al., 2003). In BM of patients with myelodysplastic syndrome (MDS), the refractory anaemia with excess of blasts (RAEB) subgroup had microvessels with a smaller calibre than the refractory anaemia (RA) and refractory anaemia with ring sideroblasts (RARS) subgroups (Korkolopoulou et al., 2001). Perez-Atayde et al. (1997) used a computer-aided three-dimensional reconstruction model of BM vascularity in BM biopsies of newly diagnosed cases of acute lymphoblastic leukaemia (ALL) and they found complex, arborising of microvessels in the leukaemic specimens compared with single, straight microvessels without branching in controls.

All tumour vessels are not equal in their ability to provide oxygen and nutrients to the tumour cells they support. Within a tumour there is an unevenly distributed tumour cell population with a varying resistance to hypoxia and with a heterogeneous ability to up-regulate HIF or to secrete angiogenic factors. This is reflected in an unevenly distributed vasculature.

Vacca et al. (2003a) studied genotypic and antigenic differences between MM-associated BM EC and the quiescent human umbilical vein (HUVEC) EC. The MM-endothelial cells highly expressed VEGFR-2, Tie-2 and CD105 (endoglin) (involved in vessel sprouting), CD34 and CD133 (suggesting recruitment of BM endothelial progenitor cells), bFGFR-2 (bFGF is secreted by MM cells and stromal cells), and aquaporin 1 (hyperpermeability) in comparison to HUVEC.

Since tumour cells can constitutively secrete angiogenic factors without physiological feed-back control, the vascularisation of the tumour may become greater than is necessary for the metabolic need (over-vascularisation) (Hlatky et al., 2002). This can have therapeutic consequences since reducing redundant microvessels with anti-angiogenic therapy will probably have less effect in reducing tumour mass.

The EC are an active component of the microenvironment and as such are involved in several steps of the homing of MM cells. For the specific homing, the MM cells have to be attracted to the bone marrow. Arriving in the sinusoids of the BM, they have to be arrested on the endothelial lining of the sinusoids.

Subsequently they have to migrate through the EC and the basement membrane in order to arrive into the extravascular compartment. In this compartment they have to adhere to extracellular matrix proteins and stromal cells in order to receive the appropriate growth stimuli. Murine BMEC produce MCP-1, which after binding on CCR2, present on both 5T2 and 5T33MM cells (this murine MM model is discussed later), induces chemo-attraction to the BM (Vanderkerken et al., 2002). Once the MM cells are attracted to the BM they have to adhere to the BMEC. Murine 5TMM cells preferentially adhere to BM EC via CD44v10 expressed on MM cells (Asosingh et al., 2001). Contact with BMEC causes up-regulation of key molecules such as CD44v6 (adhesion to BM stroma), IGF-1R (chemotaxis to the BM, migration of MM cells,) and MMP-9 (infiltration through the basement membrane) by the MM cells on 5TMM cells (Van Valckenborgh et al., 2002; Asosingh et al., 2000a; Menu et al., 2003). In the human, the up-regulation of MMP-9 in the MM cells has been demonstrated to be mediated by hepatocyte growth factor (HGF) secreted by the BMEC (Vande Broek et al., 2003).

There is recent evidence that murine BMEC express RANKL (receptor activator of NFκB) and produce osteoprotegerine (OPG) (De Leenheer et al., 2003). The RANKL/OPG system plays an important role in the development of normal osteoclasts and the system is abnormally regulated in MM resulting in bone destruction (Croucher et al., 2001; Vanderkerken et al., 2003a). MM cells decrease OPG production by the EC (De Leenheer et al., 2003).

How to measure angiogenesis in trephine biopsies

The quantification of angiogenesis in solid tumours is extensively discussed in an international consensus report (Vermeulen et al., 2002). We here address some of the methods applicable for trephine biopsies which could be used in routine work. Other non-invasive functional methods to assess the vascularity such as Doppler sonography, positron emission tomography and contrast-enhanced dynamic magnetic resonance imaging will not be addressed here, but are extensively reviewed elsewhere (Mc Donald and Choyke, 2003).

Microvessel density

Determining the microvessel density (MVD) has a prognostic value in a wide range of tumours such as invasive breast cancer (Acenero et al., 1998), colorectal cancer (Gallego et al., 2000), prostate cancer (Weidner et al., 1993), urinary bladder (Chaudhary et al., 1999), oesophageal and gastro-intestinal carcinoma, and malignant melanoma (Graham et al., 1994).

The MVD is the net result of cumulative phases of angiogenesis and angioregression. It is a misconception to assume that the MVD is a measure for the angiogenic activity at the time of biopsy (Hlatky et al., 2002).

Measuring the MVD is subjected to important factors which can cause confusion: 1) sample size; inadequate sampling can be an important source of error since the microvessels are heterogeneously distributed within the BM; 2) non-tumour related causes of angiogenesis e.g. infection, healing (previous biopsy), hypoxia from systemic origin (haemorrhagic shock); 3) type of fixative used, the process of decalcification; 4) antibody selection. Each of the immunostainings have their advantages and drawbacks (Vidal et al., 2000; Hasan et al., 2002). The FVIII staining, although specific, is not very sensitive. The megakaryocytes express FVIII. *Ulex europeus* may also label some inflammatory and neoplastic cells. The CD31 (PECAM-1) (Sheibani and Frazier, 1999) immunohistochemistry also stains platelets and megakaryocytes and some PC. An interfering feature of the CD34 in the BM is the expression of CD34 on myeloid blasts, which makes this immunostain unsuitable for the assessment of the MVD in CD34-positive AML. Pruneri et al. (2002) evaluated the MVD in MM by staining with anti-CD105 and anti-CD34 antibodies. The anti-CD34 antibody preferentially highlighted the small vessel endothelial sprouts, whereas the anti-CD105 antibody better highlighted the sinusoid-like vessels. Although the MVD was significantly higher in MM than in controls after immunostaining with either the anti-CD105 antibody or the anti-CD34 antibody, only the MVD determined with the latter had a prognostic value. In solid tumours the superiority of anti-CD105 over anti-CD34 was demonstrated in non-small cell lung (Tanaka et al., 2001) and breast carcinomas (Kumar et al., 1999). Thrombomodulin is constitutively highly expressed by EC and can also be used to highlight microvessels (Pulé et al., 2002); 5) training and experience of the investigator influences the identification of the vascular hot spot; and 6) methods of counting: since the microvessels are unevenly distributed, different methods have been used to evaluate a representative tumour area. It has been suggested that EC proliferation is particularly active in the highly vascularised regions ("hot spots") (Weidner et al., 1991). One method often used is the quantification of the MVD in the hot spot. A limitation of this method is the subjectivity of hot spot determination. Another method used for solid tumours is the Chalkley point overlap morphometry technique (Chalkley, 1943; Vermeulen et al., 2002). This method uses an eyepiece graticule that contains a number of grid points. By turning the grid the maximum number of grid points has to be superimposed on the microvessels (within the hot spot). This number reflects the relative area occupied by the microvessels rather than the vessel density. The main advantage of this method is that no decision has to be made by the observer as to whether two adjacent immunostained cross-sections belong to one or to two separate blood vessels, and thus reduces the subjectivity. The Chalkley technique has been used in studies of breast cancer patients and the count proved to be a significant and independent prognostic factor. In the BM environment,

however, this method can give discrepant results to those obtained by MVD determination in the hot spots: normal sinusoids have an open lumen and it is easy to superimpose more grid points on such vessels than on the small newly-formed slit-like vessels in haematological malignancies. The observation that newly-formed microvessels are smaller than the pre-existing sinusoids implies that an increase in MVD will be stronger than the increase in the Chalkley count, the latter being an estimate of the microvessel area.

The MVD is the net result of cumulative phases of angiogenesis and angioregression, and therefore can not predict whether a patient will respond to anti-angiogenic therapy. Oxygen and nutrient consumption of a tumour determine which intercapillary distance is compatible with viability. This does not however imply that a tumour with a low MVD (and a high intercapillary distance) is less dependent on oxygen and nutrient supply. Another limitation of the MVD is that its value does not take into account the functionality of the vessels. Reducing microvessels in a condition of over-vascularisation (cf. supra), inhibiting hypoxic vessels or inhibiting vessels with a stagnating blood flow, will probably be less effective. During anti-angiogenic therapy the MVD is difficult to interpret, since the evolution of the MVD under anti-angiogenic therapy is dependent on several factors. Assuming that the therapy succeeds in reducing the microvasculature, a reduction in MVD will be observed if the decrease in MVD is stronger than the tumour mass reduction The MVD will remain constant if the decrease in microvasculature parallels the decrease in tumour mass. Even an increase in MVD can theoretically be compatible with a successful therapy in the situation where the tumour mass reduction is stronger than the decrease in MVD (Folkman, 2001).

Endothelial cell proliferation

To have a better idea of the ongoing angiogenesis at the time of biopsy the endothelial cell proliferation (ECP) fraction can be determined on the basis of a double immunostaining for CD34 and Ki-67: CD34 will highlight the EC of the microvessels while Ki-67 will stain the nuclei of the proliferating EC. The ECP fraction of quiescent adult vasculature is 0.01% (Carmeliet and Jain, 2000). This is not unexpected since these EC have a life span of several hundred days. The EC of tumour-associated vessels have a turnover of around 5 days. In haematological malignancies we observed an ECP varying between 0 and 7% (De Raeve et al., 2004).

Angiogenesis in multiple myeloma

Several reports have found independently that increased MVD is an adverse prognostic marker for MM (Vacca et al., 1994; Rajkumar et al., 2000; Munshi and Wilson, 2001). There is a correlation between the MVD, the clinical stage and cytological grade (Xu et al., 2002).

A decreased MVD in MM patients achieving complete remission after chemotherapy has been reported by one study (Sezer et al., 2001b), but was not observed in another study (Rajkumar, 1999), where MVD was compared before and after chemotherapy followed by stem cell transplantation. The MVD of BM in patients with active MM (Fig. 1b) is significantly higher than in patients with inactive MM or patients with monoclonal gammopathy of undetermined significance (MGUS) (Vacca et al., 1994). These observations suggest that active MM may represent the "vascular phase" of plasma cell tumours, and non-active MM and MGUS the "prevascular phase" (Fig. 1a). Moreover, since BM angiogenesis and labelling index (LI) are closely associated with the phases of MM activity, and since the LI is a prognostic factor, it may well be that the MGUS and non-active MM are at risk of progression towards active MM if the BM shows angiogenesis (Ribatti et al., 2003b). MM cells of patients with active MM express VEGF (Dankbar et al., 2000), bFGF (Vacca et al., 2003a), HGF (Borset et al., 1996) and Ang-1 (Giuliani et al., 2003). VEGF induces proliferation and triggers migration of human MM cells via an autocrine loop. VEGF isoform 165 activates at least two pathways in MM: the Raf-1-MEK-extracellular signal-regulated protein kinase (ERK) pathway mediating proliferation, and the protein kinase C (PKC)-dependent cascade associated with migration (Podar et al., 2001). In addition VEGF is involved in several paracrine loops which trigger growth of MM. MM cells secrete VEGF-A which stimulates proliferation and chemotaxis in EC via VEGFR-2 and in stromal cells via VEGFR-1. Residual stromal cells secrete VEGF-C and VEGF-D which trigger plasma cell proliferation via VEGFR-3 (Vacca et al., 2003b). Both splice variants VEGF165 and VEGF121 are secreted by MM cells and stimulate the expression of IL-6 by BM EC and BM stromal cells. In turn, IL-6 stimulates the expression of both VEGF splice variants by MM cells (Dankbar et al., 2000). Along with IL-6, other cytokines and growth factors, such as bFGF (Bisping et al., 2002), IL-1ß, PDGF, insulin-like growth factor, TGF-ß, TNF-α, and keratinocyte growth factor, have been reported to stimulate VEGF expression (Dankbar et al., 2000).

Serum HGF and serum VEGF levels are increased in MM (Iwasaki et al., 2002), but do not correlate with disease severity, as indicated by the stage of disease and ß2-microglobulin (ß2M) (Sezer et al., 2001a).

MMPs are also involved in the angiogenesis in MM. Of the 24 different MMPs known to be involved in the progression of malignancies, MMP-2, -7, -8, -9 and -13 (Barillé et al., 1999; Wahlgren et al., 2001; Vacca et al., 2003a) have been demonstrated in human MM cell lines and patients. MMP-2 is also secreted by BM stroma (Barillé et al., 1997). The secretion of MMP-9 by MM cells is dependent on the tumour-stroma interaction (Barillé et al., 1997). Moreover, the MMP-2 secretion is more pronounced in patients with active MM than in those with non-active MM and with MGUS (Vacca et

al., 2003a). MMPs are able to degrade many components of the extracellular matrix (ECP) thereby promoting invasion, metastasis and angiogenesis (Hiraoka et al., 1998; Bergers et al., 2000; Egeblad and Werb, 2002). During the degradation of the ECM, matrix-bound growth factors and angiogenic factors are released (Kalluri et al., 2003). The capillary basement membrane is composed of type IV collagen, laminin, heparan-sulphate proteoglycans, perlecans, nidogen/entactin SPARC/BM-40/osteopontin, type XV collagen and type XVIII collagen (Kalluri et al., 2003). The structure of the capillary BM is complex with a high level of cross-linking. When the basement membrane is being disassembled some of the sequestered proteins (such as VEGF and bFGF) are released or different domains (cryptic domains of partially degraded collagens) of proteins with angiogenic potential are exposed. In addition, the basement membrane degradation liberates the EC from their cell-surface anchors (integrins) enabling the EC to migrate and to proliferate (vascular sprouting). As the basement membrane degradation reaches completion, the remaining MMP-resistant products, such as endostatin, arrestin, constatin and tumstatin have anti-angiogenic effects. The MMP-induced degradation of the basement membrane therefore initially promotes angiogenesis and later on inhibits angiogenesis (Kalluri et al., 2003). Besides their role in angiogenesis the MMPs participate in the recruitment of osteoclasts to the sites of resorption (Sato et al., 1998) and play a role in bone resorption (Holliday et al., 1997; Everts et al., 1998). Under physiological conditions the activity of MMPs is tightly controlled by endogenous tissue inhibitors of MMP (TIMP). Synthetic broad-spectrum MMP inhibitors have been used for cancer therapy with disappointing results. As a major side-effect musculo-skeletal pain, which was attributed to the inhibitory effect on MMP-1, has been reported (Coussens et al., 2002).

For the study of the pathophysiology of MM in general and the angiogenesis in particular, the murine 5T33MM and 5T2MM models have been developed (Radl et al., 1988; Asosingh et al., 2000b; Vanderkerken et al., 2003b). Both 5TMM cells originated from elderly C57Bl/KaLwRij mice that developed spontaneously MM. The 5TMM models are propagated in vivo by isolating the BM of diseased mice followed by i.v. transfer of isolated MM cells into young syngeneic mice. The 5T2MM model is characterised by a moderate growth and the development of osteolytic lesions while the 5T33MM model is more aggressive and representative for the human plasmablastic MM (Vanderkerken et al., 1997). In both the 5T2MM and the 5T33MM tumour growth is associated with an increased angiogenesis (Van Valckenborgh et al., 2002). The 5T33MM cells express VEGF-A, bFGF, PDGF (A,B,C,D) (Van Riet et al., 2003) and Ang-1. Upon interaction with BMEC, the 5T33MMvt cells secrete MMP-9 (Van Valckenborgh et al., 2002). In the 5T2MM model experiments are underway with a broad spectrum
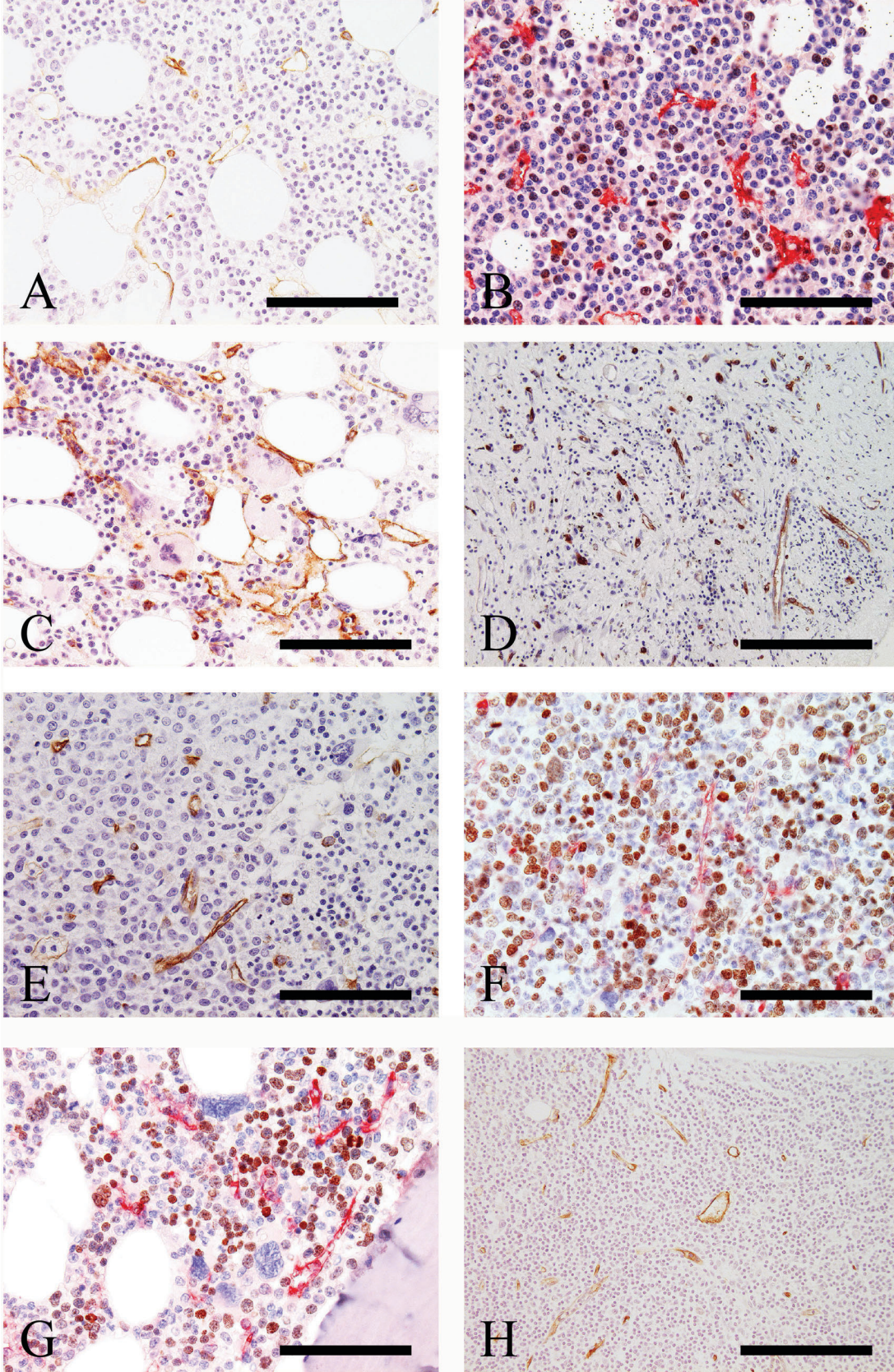
Fig. 1. Sections of paraffin-embedded and decalcified trephine biopsies. **A.** CD34 immunostaining of a MGUS. There is no increased MVD. The microvessels (sinusoids) are totally comparable to sinusoids of control BM. The thin endothelial lining is weakly positive for CD34. **B.** Ki-67/CD34 immunostaining of a diffusely growing MM. The nuclei of proliferating cells stain brown with the Ki-67 immunostaining. The endothelial cells stain red with the CD34 immunostaining. The MVD is increased. The microvessels have slit-like lumina and the endothelial lining is strongly positive for CD34. Proliferating endothelial cells can be recognised by a brown-stained nucleus and a red-stained cell membrane (not present in this figure). **C.** CD34 immunostaining of an IMF in a cellular phase. There is an increased MVD. The abnormal megakaryocytes are often in close contact with the microvessel. Among the CMPD, IMF has the highest MVD. **D.** CD34 immunostaining of an IMF in a fibrotic phase. The MVD is markedly increased. Rare microvessels have an open lumen and are weakly stained for CD34. The majority of the microvessels have a compressed lumen and strongly express CD34. **E.** CD34 immunostaining of a CML in chronic phase. Microvessels with an open lumen and microvessels with a slit-like lumen are both present. **F.** Ki-67/CD34 immunostaining of a CML in chronic phase. **G.** Ki-67/CD34 immunostaining of an ET. The cellularity is moderately increased. Some microvessels with slit-like lumen are present. The MVD in ET is increased compared to control BM and BM from patients with reactive thrombocytosis. **H.** CD34 immunostaining of a CLL. Despite the diffuse infiltration there is no increase in MVD. Bars: A-C, E-G, 100 μm; D, H, 200 μm.

MMP-inhibitor SC-964 and the first results show a reduction in tumour growth, a direct anti-angiogenic effect and a reduction in osteolytic lesions (Van Valckenborgh et al., 2003).

Another animal model which proved to be useful in the study of angiogenesis in MM is the severe combined immunodeficiency (SCID)-hu model of human MM (Urachima et al., 1997) in which human fetal bone grafts are implanted bilaterally in the flanks of SCID mice. In this model it was demonstrated that thalidomide and immunomodulatory derivatives of thalidomide mediate both anti-MM activity and anti-angiogenesis (Lentzsch et al., 2003).

Angiogenesis in myelodysplastic syndrome and acute myeloid leukaemia

In myelodysplastic syndromes the MVD is significantly increased compared to normal controls. Among the French-American British (FAB) subtypes, the MVD is significantly higher in RAEB-t, CMML, and MDS with fibrosis compared to RA, RARS and RAEB subsets (Pruneri et al., 1999). In multivariate analysis, MVD has prognostic power together with age and haemoglobin level (Korkolopoulou et al., 2001). In AML there is an increased angiogenesis in active disease compared to normal BM (Hussong et al., 2000; Padro et al., 2000; Aguayo et al., 2000a; Kini et al., 2001). A decrease, or even levels back to the control level, are observed in patients after achieving complete remission (de Bont et al., 2001b). Rimsza et al. (2002) divided AML samples into two broad, therapeutically relevant prognostic groups: favourable/intermediate and unfavourable and observed that the AML cases with unfavourable prognostic features were more likely to enhance EC proliferation in vitro than cases with favourable/intermediate prognosis. They suggested that the complex karyotypic and molecular genetic changes in the former would alter the expression of angioregulatory molecules such as VEGF. Indeed, AML blasts can express VEGF (Fiedler, 1997) and aberrant VEGF secretion and aberrant expression of VEGFR-2 have been shown to play an important role in leukaemia cell survival (de Bont et al., 2002). The VEGF secreted by the leukaemic cells can support leukaemic cell survival directly by autocrine stimulation of the VEGFR-2 (Dias et al., 2000) and via an EC-dependent paracrine pathway by triggered secretion of GM-CSF, G-CSF, Il-8 and M-CSF by the EC (Bellamy et al., 1999). The in vitro expression of cellular VEGF was reported to be an independent prognostic factor for overall survival in a high-risk subgroup of patients (de Bont et al., 2002). There is a significant relationship between increasing cellular VEGF levels and shorter survival as well as shorter disease-free survival (Aguayo et al., 1999). In acute promyelocytic leukaemia there is an increased production of VEGF which can be inhibited by all-transretinoic acid therapy (Kini et al., 2001). In contrast to AML, the increased plasma VEGF levels in MDS do not have a prognostic value (Aguayo et al., 2002).

AML cells can produce other angiogenic factors such as IL-8 (Tobler et al., 1993), bFGF (Hussong et al. 2000), Ang-1 (Müller et al., 2002) , MMP-2 and MMP-9 (de Bont et al., 2001b). MMP-2 secretion is frequently found in AML cells, but is absent in normal haematopoietic progenitor cells (Janowska-Wieczorek et al., 1999) while MMP-9 is expressed in AML cells, normal mononuclear cells and CD34+ progenitor cells (Ries et al., 1999). In CMML the plasma levels of VEGF, HGF and TNFα are strongly increased (Aguayo et al., 2000a). Plasma levels of TNFα are not significantly increased in MDS and AML (Aguayo et al., 2000a).

Angiogenesis in acute lymphoblastic leukaemia

An increased MVD has initially been observed in ALL in a study of 40 children (Perez-Atayde et al., 1997) and has been confirmed subsequently (Aguayo et al., 2000a; Pulé et al., 2002). When dividing microvessels into either small (smallest diameter <17 μm) and large (smallest diameter >17 μm), the difference in total MVD count between control patients and AML patients could be entirely attributed to differences in small microvessels (Pulé et al., 2002), i.e. the MVD for large microvessels showed no increase in the leukaemic sample. At remission, small microvessels dropped substantially. This observation may suggest that the smaller microvessels represent "buds" or "sprouts" involved in active vascular remodelling. At presentation there is a broad spectrum of MVD. No correlation has been observed between MVD and age, sex, cytogenetics, and immunophenotype (Pulé et al., 2002). The MVD in ALL has no prognostic value and is no indicator for relapse (Pulé et al., 2002).

In ALL there are increased plasma levels of HGF, TNFα and bFGF, but not of VEGF (Aguayo et al., 2000a). MMP secretion has been demonstrated in different lymphoblastoid cell lines such as Burkitt's lymphoma, B-cell lymphoblastic leukaemia and T-cell lymphoblastic leukaemia (Vacca et al., 1998).

Angiogenesis in chronic lymphocytic leukaemia

Kini et al. (2000) found a significantly increased MVD in 12 B-CLL patients. They used the CD34 monoclonal antibody and found that the MVD correlated with the cellularity and the stage. In a study of 129 patients the MVD was counted in the hot spots on the basis of FVIIII-RA immunostaining and no increase in MVD was observed (Aguayo et al., 1999) (Fig. 1h).

Molica et al. (2002a) measured the microvessel area and could distinguish two subpopulations within the CLL population: those with a microvessel area ≥ 0.009 mm^2 had a significantly shorter survival than those with a microvessel area <0.009 mm^2. The microvessel area

correlated with the VEGF expression by B-CLL cells. Intracellular levels of VEGF in CLL are of prognostic significance (Aguayo et al., 2000b). A brisk increase in the detectable mRNA levels of VEGF and protein secretion has been observed when B-CLL cells were exposed to hypoxia (Chen et al., 2000). The elevation of VEGF in hypoxic conditions was variable from clone to clone but did suggest that under the appropriate environmental conditions CLL cells were capable of releasing increased levels of VEGF.

Serum levels of VEGF were found to be of prognostic value, but did not correlate with tumour angiogenesis. As discussed previously, the presence of many sources of sVEGF, such as stromal cells, platelets, and EC, and the involvement of several angiogenic and anti-angiogenic factors, may account for this observation. Examining the serum levels of VEGF (sVEGF) in combination with the serum levels of ß2M was revealed to be a good prognostic tool (Molica et al., 2002b): the median progression-free survival of patients who had both sVEGF and sß2M levels above 203 pg/ml and 2.73 mg/L respectively was 13 months, while patients who had both sVEGF and sß2M below 203 pg/ml and 2.73 mg/L had a median progression-free survival longer than 40 months.

B-CLL cells express both VEGFR-1 (flt-1) and VEGFR-2 (kdr) indicating that VEGF may also have a direct autocrine growth stimulatory function (Kay et al., 2002). Levels of VEGFR-1 correlate with white blood counts and with levels of cellular VEGF (Aguayo et al., 2001). Another receptor expressed on CLL cells is the Tie-1 receptor tyrosine kinase, which correlated with white blood cell count. When evaluated in early-stage disease, VEGFR-1 and Tie-1 did not correlate with survival (Aguayo et al., 2001).

There is evidence that bFGF acts as a survival factor for CLL (Gabrilove, 2001): higher intracellular bFGF levels in B-CLL cells in patients with high-risk disease and resistance to fludarabine has been observed (Menzel et al., 1996), and bFGF upregulates bcl-2 expression, which induces resistance to apoptosis (Konig et al., 1997). bFGF serum levels are generally high in early B-CLL but do not have prognostic power (Molica et al., 2002b). CLL cells are also known to secrete the pro-angiogenic factor TGFß (Schuler et al., 1999) and MMP-9 (Bauvois et al., 2002). Besides the secretion of pro-angiogenic molecules the anti-angiogenic factor thrombospondin-1 is consistently secreted by the B-CLL cells; to a lesser extent the anti-angiogenic factors endostatin and interferon α are also secreted (Kay et al., 2002). Under hypoxic conditions the levels of mRNA of thrombospondin-1 decrease (Kay et al., 2002). Thus, there are both pro- and anti-angiogenic molecules secreted by the CLL cell clones and an imbalance in the production between these factors could induce an angiogenic switch. The factors responsible for such an imbalance could be a hypoxic environment and/or genetic mutations, such as p53 mutations, which are documented in progressive CLL (Callet-Bauchu et al.,

1999).

Angiogenesis in chronic myeloproliferative disease

In CMPD a significantly increased MVD has been observed in chronic myelocytic leukaemia (CML) (Fig 1e,f) and in idiopathic myelofibrosis (IMF) (Fig. 1c,d) and a moderate increase in polycythaemia vera (Lundberg et al., 2000). Aguayo et al. (2000a) observed a higher vascular area and higher plasma levels of VEGF in CML compared to AML. In CML there was an increased plasma concentration of bFGF, HGF and TNFα. In a multivariate analysis (Korkolopoulou et al., 2003) of 52 patients with CML, the microvessel area was related to progression-free survival, whereas both MVD and microvessel area were significant prognosticators for overall survival. A positive correlation emerged between BM fibrosis and MVD. CML cells express Ang-1 and its receptor Tie-2 suggesting that these angiogenic molecules might be involved in an autocrine stimulatory pathway (Müller et al., 2002). The Tie-1 tyrosine kinase receptor protein levels in BM samples of CML patients are significantly higher when compared to control patients (Verstovsek et al., 2002). Tie-1 BM levels are a predictor of survival in early chronic phase CML independent of risk group, spleen size, age, and haemoglobin and basophil count (Verstovsek et al., 2002).

Using the pathognomonic bcr-abl-fusion gene as a genetic marker present in virtually all BM-derived cells of patients with CML, Gunsilius (2003) was able to show that EC belong to the malignant cell clone, since they also contained the bcr-abl-fusion gene. These data suggested that CML arises from a haemangioblastic progenitor cell, the progeny of which are malignant blood cells, a genotypically clonal EC. In addition, this study provided evidence for the existence of an haemangioblast in the BM of adults and for the existence of post-natal vasculogenesis.

The angiogenesis in patients with IMF is much more pronounced than in those with either PV or essential thrombocythaemia (ET) (Fig. 1g) (Mesa et al., 2000a). The increase in MVD in IMF correlates with cellularity and megakaryocyte clumping (Mesa et al., 2000a) and, in a multivariate analysis, correlated with increased spleen size and overall survival (Mesa et al., 2000a). The clonal proliferation of megakaryocytes in IMF is accompanied by the abnormal release of cytokines including angiogenic factors resulting in an excessive stromal reaction (Reilly, 1997) and an increase in BM vascularity (Thiele et al., 1992). Implicated cytokines include TGF-ß (Martyre et al., 1994), bFGF (Martyre et al., 1997; Chou et al., 2002), platelet-derived growth factor (PDGF) and VEGF. The constitutive expression and secretion of VEGF in human megakaryocytes can be increased by either a paracrine or an autocrine mechanism (Mohle et al., 1997; Bellamy et al., 1999).

In PV there are increased sVEGF levels in 90 % of the patients and splenomegaly is associated with an

increase in sVEGF levels (Murphy et al., 2002). In IMF there is an increased microvascular TGF-ßRI up-regulation (Chou et al., 2002). Cyclooxygenase (Cox)-2, the inducible form of Cox that converts arachidonic acid to prostaglandins, and Tie-2 are immunohistochemically detectable in megakaryocytes of normal, CML and PV patients (Zetterberg et al., 2003). Whether these molecules are abnormally regulated in megakaryocytes from patients with myeloproliferative disorders remains to be elucidated.

Mesa et al. (2000b) determined the MVD on the basis of a CD34 stain in 164 ET patients and found an increased MVD compared to either normal controls or patients with reactive thrombocytosis.

Therapeutic considerations and conclusion

One of the major advantages of anti-angiogenic therapy is that it will not generate drug-resistant cell populations, given that the malignant cells are not the direct target.

Since more and more angiogenic pathways are unravelled, it becomes clear that there is a redundancy of mechanisms by which the tumour succeeds in switching on angiogenesis. Targeting one angiogenic factor has proven to be insufficient to achieve a complete remission.

Combination of a VEGF-R inhibitor together with a receptor tyrosine kinase inhibitor targeting PDGF-R activity could be more efficacious even in late-stage tumours (Bergers et al., 2003). By targeting the PDGFR-ß signalling, the interaction between EC and pericytes is disturbed which destabilises the tumour vessels and renders them more vulnerable to anti-VEGF therapy. A triple-action inhibitor such as SU6668 is a potent small-molecule inhibitor of the receptor tyrosine kinases VEGFR-2, bFGFR and PDGFR (Hoekman, 2001). In addition SU6668 has an inhibitory activity on the stem cell factor (SCF) receptor, c-kit, which is structurally related to the former receptors and is expressed on 60 to 80% of the leukaemic blasts of AML patients. c-kit plays a role in promoting the growth of leukaemic cells. Hence, there are three mechanisms by which SU6668 may be beneficial for AML patients. First, inhibition of c-kit may lead to a reduction in blast cell proliferation. Second, by inhibition of angiogenesis and third, by reduction of the EC as paracrine sources of growth factors (such as SCF) (Smolich et al., 2001).

Recognition of the significance of stromal cell-tumour cell interactions has spurred an intensive research effort to develop targeted molecular therapies that can disrupt these interactions. Novel agents that target both the tumour and the microenvironment offer promising perspectives in the field of MM (Anderson, 2003; Anderson et al., 2003). For example, the immunomodulatory derivatives of thalidomide as well as the proteasome inhibitor PS-341 (bortezomid) (Hideshima et al., 2001, 2003) induce apoptosis of MM cells, block the production of cytokines involved in growth, survival, drug resistance (IL-6, VEGF, TNFα) and migration, inhibit angiogenesis and impede the interaction between MM cells and fibronectin and between MM cells and the BM stromal cells. These agents also stimulate host anti-tumour immunity by expanding the patient NK-cell number and function against MM cells and by stimulating T-cell proliferation and production of IL-2 and IFN-γ (Davies et al., 2001). With the use of these agents, a state of tumour dormancy will probably be attained.

The challenge with anti-angiogenic therapy will be to find the appropriate combinations of these drugs including the appropriate timing. For example, the MMP inhibitors are most effective in the initial phase of the disease, since the MMPs are involved in the angiogenic switch (Bergers et al., 2000). Judiciously applied anti-angiogenic therapy can be applied concurrently with conventional chemotherapy or radiotherapy (Jain, 2001; Lee et al., 2000), combining two different modes of action to affect tumour growth. The anti-angiogenic therapy, by restoring the balance of pro- and anti-angiogenic cytokines, will reduce the disorganisation of the tumour vasculature and facilitate the oxygen supply and the delivery of therapeutics. In addition, the anti-angiogenic therapy will reduce the EC as a paracrine source of growth factors.

On the other hand, some of the conventional chemotherapeutic agents, such as cyclophosphamide, have been shown to have anti-angiogenic capabilities by changing the dosage scheduling (Browder et al., 2000); in classical chemotherapy dosage schedules the highest survivable dose is chosen followed by a treatment-free interval to permit recovery of normal host cells, such as rapidly growing haematopoietic progenitors. In this same interval the vascular EC can also resume growth, which could support re-growth of tumour cells and could increase the risk of the emergence of drug-resistant tumour cells. To more effectively suppress the re-growth of the tumour-associated EC, the conventional chemotherapeutic agent is administered at shorter intervals without interruption. The combination of this therapy together with other anti-angiogenic agents could be beneficial in slowly growing haematological malignancies.

For the selection of patients and the evaluation of treatment efficacy one should realise that the MVD in se is not accurate enough. By judging therapy efficacy on the basis of classical clinical parameters (clinical examination, imaging, tumour markers,…) one should be aware of the fact that apoptosis of EC and decrease of the vasculature will usually precede the reduction in tumour mass. Moreover, the fact that not all vessels are functionally equal, the fact that the blood flow is not uniform in time and space and the fact that tumours can constitutively over-express angiogenic factors (resulting in over-vascularisation) (Hlatky et al., 2002), makes the evaluation of treatment efficacy even more difficult. For the pathologist who wants to have an idea of ongoing angiogenesis at the time of biopsy, the determination of

the ECP fraction by means of double immunostained sections is an option, provided that the sample is representative.

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