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The Role of Microenvironment in the Control of Tumor Angiogenesis

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List of Abbreviations

ALDH1	Aldehyde dehydrogenase 1
AML	Acute myeloid leukemia
ANG-1	Angiopoietin-1
APL	Acute promyelocytic leukemia
A-SMA	Alpha smooth actin
ATRA	All trans retinoic acid
BM	Bone marrow
BMP	Bone morphogenetic protein
CAM	Chorioallantoic membrane
CCL5	Chemokine (C-C motif) ligand-5
COX-2	Cycloxygenase-2
CSC	Cancer stem cell
CSF	Colony stimulating factor
CSF-1R	CSF-1 receptor
CXCL	Chemokine (C-X-C motif) ligand
DLBCL	Diffuse large B-cell lymphoma
ECs	Endothelial cells
ECM	Extracellular matrix
EDG-1	Endothelial differentiation gene-1
EGF	Epidermal growth factor
EMT	Epithelial mesenchymal transition
eNOS	Endothelial nitric oxide synthase
EPCs	Endothelial precursor or progenitor cells
ESA	Epidermal surface antigen
FAP	Fibroblast activating protein
FDA	Food and Drug Administration
FGF-2	Fibroblast growth factor-2
FSP-1	Fibroblast specific protein-1
GCSF	Granulocyte colony stimulating factor
GFP	Green fluorescent protein

GIST	Gastrointestinal stroma tumors
GM-CSF	Granulocyte macrophage colony stimulating factor
GSC	Glioblastoma stem-like cells
HCs	Hematopoietic Cells
HGF	Hepatocyte growth factor
HIFs	Hypoxia inducible factors
HPCs	Hematopoietic precursor cells
HPV	Human papilloma virus
HRG	Histidine-rich glycoprotein
HSCs	Hematopoietic stem cells
ICAM	Intercellular cellular adhesion molecule
IFN	Interferon
IGF-1	Insulin-like growth factor-1
IL	Interleukin
IMG	Intussusceptive microvascular growth
iNOS	Inducible nitric oxide synthase
LT-CSC	Long term CSC
M-CSF	Macrophage-colony stimulating factor
mTOR	Mammalian target of rapamycin
MAPCs	Multipotent adult progenitor cells
MCP	Monocyte chemotactic protein
MDSCs	Myeloid derived suppressor cells
MET	Mesenchymal epithelial transition
MGUS	Monoclonal gammopathy of undetermined significance
MIP-2	Macrophage inflammatory protein-2
MM	Multiple myeloma
MMP-9	Matrix metalloproteinase-9
MSCs	Mesenchymal stem cells
MT1-MMP	Membrane type-1-MMP
mTOR	Mammalian target of rapamycin
NAID	Nonsteroidal anti-inflammatory drugs
NECA	N-methyl carboxamide adenosine
NG2	Nerve/glial antigen-2
NGF	Nerve growth factor
NK	Natural killer
NOD-SCID	Non obese diabetic/severe combined immunodeficiency
NSAIDs	Non-steroidal anti-inflammatory drugs
NSCLS	Non small cell lung cancer
OPN	Osteopontin
PlGF-1	Placental growth factor-1
PA	Plasminogen activator
PAR	Protease activated receptor
PC	Pericytes
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor

PGE2	Prostaglandin E2
PSMA	Prostate specific membrane antigen
PTX-3	Pantraxin-3
PyMT	Polyoma middle-T
RA	Retinoic acid
RNI	Reactive nitrogen intermediate
ROS	Reactive oxygen species
RTKIs	Receptor tyrosine kinase inhibitors
SCF	Stem cell factor
SDF-1	Stromal cell derived factor 1
ST-CSC	Short term CSC
TAF	Tumor angiogenesis factor
TAFs	Tumor associated fibroblast
TAM	Tumor associated macrophages
TEMs	Tie2-expressing monocytes
TGF-β	Transforming growth factor beta
TIMP-3	Tissue inhibitor of matrix metalloproteinase-3
TNF-α	Tumor necrosis factor alpha
TRAMP	Transgenic adenocarcinoma of the mouse prostate
Treg	T regulatory
TSP-1	Thrombospondin-1
VCAM	Vascular cellular adhesion molecule
VE-cadherin	Vascular endothelia cadherin
VEGF	Vascular endothelial growth factor
VEGFR1	Vascular endothelial growth factor receptor 1
VVO	Vesciculo vacuolar organelles
VWF	Von Willebrand factor

Chapter 1
Tumor Microenvironment

Abstract Tumor microenvironment plays an important role in the initiation and progression of tumors. Studies on neoplastic transformation have focused on events that occur within tumor cells, and have addressed the microenvironment of tumor cells documenting its importance in supporting tumor progression. Before metastatic dissemination, primary tumors secrete factors that contribute to the development of a pre-metastatic niche, rendering the microenvironment more receptive to tumor growth.

Keywords Bone marrow microenvironment • Endothelial cells • Extracellular matrix • Epithelial mesenchymal transition • Fibroblasts • Hematopoietic stem cells • Hypoxia • Hypoxia inducible factors • Macrophages • Mast cells • Mesenchymal stem cells • Metastasis • Osteoblast niche • Stromal cells • Tumor microenvironment • Vascular niche

Douglas Hanahan and Robert A. Weinberg (2000, 2011) (Figs. 1.1 and 1.2) defined the hallmarks of cancer that encompass key biological capabilities including sustained proliferation, evasion of growth suppression, death resistance, replicative immortality, angiogenesis, invasion, dysregulation of cellular energetic, avoidance of immune destruction and chronic inflammation.

The final emerging cancer hallmark is metabolic reprogramming of tumor cells. Termed Warburg effect, tumor cells shift from predominantly aerobic glycolysis toward anaerobic glycolysis. Even in the presence of high oxygen tension, tumor cells exploit the anaerobic metabolic process to facilitate rapid cell division (Gillies et al. 2008).

Tumor mass dormancy or angiogenic dormancy occurs when proliferation is balanced by apoptosis because of a lack of vasculature and limited supply of nutrients and oxygen. Re-establishment of vasculature is favored by hematopoietic precursor cells (HPCs) and endothelial precursor cells (EPCs) expressing vascular endothelial growth factor receptors (VEGFRs) and by dendritic cell precursors that can differentiate into endothelial-like cells. Once tumor cells overcome dormancy, they become receptive to other cells which further support their expression.

Tumor microenvironment plays an important role in the initiation and progression of tumors (Balkwill and Mantovani 2002; Hussain and Harris 2007). Studies on neoplastic transformation have focused on events that occur within transformed

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Fig. 1.1 A portrait of Douglas Hanahan. In 2000, Hanahan and Weinberg published a classic paper entitled "The Hallmarks of Cancer" in the journal Cell. This classic paper was updated in 2011 to add two additional "emerging" hallmarks"

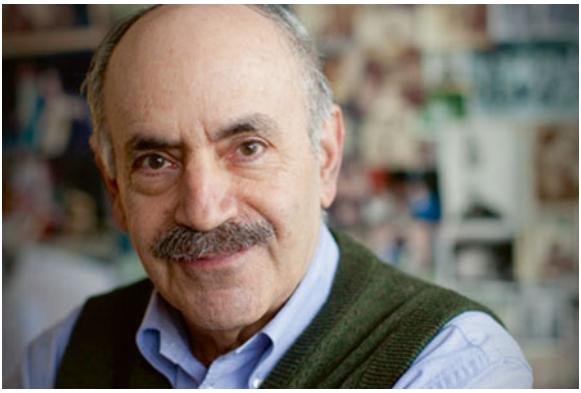

Fig. 1.2 A portrait of Robert Weinberg

cells, and have addressed the microenvironment of tumor cells documenting its importance in supporting tumor progression. The pathogenesis of most cancers includes complex and mutual interactions that affect the number and phenotype of the tumor cells and various normal stromal cells, and intricate tumor-microenvironmental interactions are increasingly recognized as critical features of several neoplasias.

Tumors are often hypoxic in spite of high vascularization due to the poor structure and functionality of tumor blood vessels. Hypoxia in tumors develops in form of chronic hypoxia, resulting from long diffusion distances between perfused tumor vessels and/or of acute hypoxia, resulting from a transient collapse of tumor vessels. Hypoxia inducible factors (HIFs) are activated in these series of events. Intratumoral hypoxia is a main cause of high reactive oxygen species (ROS) formation within the tumor cells (Guzy and Schumacker 2006) and also is coupled to pathological tumor cell metabolism and acidosis (Denko 2008). Hypoxic tumor cells have the ability to create an immunosuppressive microenvironment. Through the ability to produce HIF-1 alpha (HIF-1α), tumor necrosis factor alpha (TNF-α) and stromal cell derived factor-1 (SDF-1), hypoxic tumor cells produce the homing of $CD45^+$ myeloid cells to tumor site, including subpopulations defined by the expression of Tie-2,

Table 1.1 Pro-angiogenic factors induced by hypoxia via NF-kB pathway

NF-kB pathway
IL-1β, IL-6, IL-8
COX-2
TNF-α
MIP-2
ICAM
VCAM
CCL-5
iNOS

Table 1.2 Genes activated by hypoxia inducible factor-1 alpha

Involved in angiogenesis
VEGF
PlGF
VEGFR-1
Involved in endothelial cell proliferation and migration
VEGF
PlGF
FGF-2
CXCL12/CXCR4
PDGF
Involved in pericyte recruitment
PDGF
Ang-1
Involved in changes in vascular permeability
VEGF
VEGFR-1
Ang-1

VEGFR-1, and/or CD11b, as well as tumor associated macrophages (TAMs) (Du et al. 2008; Mantovani et al. 2008).

Stimulation of angiogenesis in the context of ongoing inflammation is usually related to hypoxia. HIF-NF-kB cross-talk present a basal molecular mechanism influencing inflammatory angiogenesis. NF-kB is a direct modulator of HIF-1α expression, as NF-kB binding site is present in HIF-1α promoter region (Table 1.1).

Hypoxia mediates immune cells recruitment and these cells concentrate at the tumor periphery, while in the tumor core hypoxia contributes to cancer cell escape by providing an aggressive selection for stem-like tumor cells that migrate away to the tumor margin. Hypoxic areas are refractory to chemo and radiotherapy and contribute to select tumor populations able to survive in poorly oxygenated niches and escape to metastatic sites and pro-angiogenic cancer stem cells (CSCs). HIF-1α acts as a survival factor for cancer cells by activating the transcription of genes involved in angiogenesis, proliferation and migration of endothelial cells (ECs), pericyte recruitment, and changes in vascular permeability (Table 1.2). Persistent extravasation

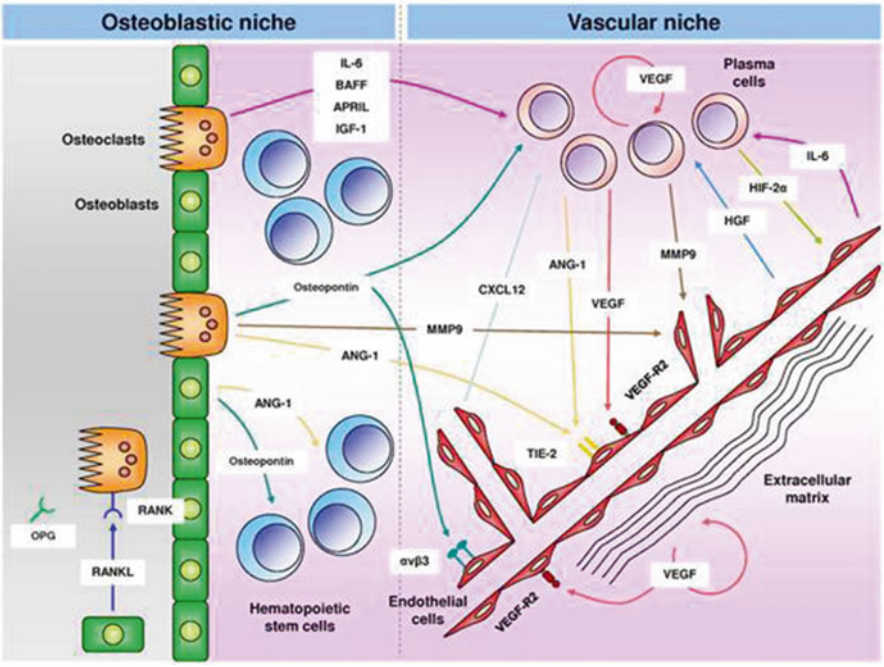

Fig. 1.3 Interactions occurring in the bone marrow niches in multiple myeloma. The non-hematopoietic cells contribute to formation of two specialized niches: an "osteoblastic niche" which is closed to endosteum, and a "vascular niche" which is closed to the bone marrow vasculature (Reproduced from Ribatti et al. (2014))

of cells enables activation of angiogenic response due to the constant and abundant release of inflammatory stimuli, which are also pro-angiogenic molecules or stimulate generation of such factors.

Hypoxia can increase the invasive potential of cancer cells by inducing the production of pro-migratory proteins (e.g. SDF-1α and hepatocyte growth factor, HGF) and pro-invasive extracellular matrix proteins (Semenza 2014).

Bone marrow (BM) microenvironment comprises hematopoietic stem cells (HSCs) and non-hematopoietic cells (HCs). HSCs give rise to all the blood cell types of the myeloid and lymphoid lineages (Krause 2002). The non-hematopoietic cells include ECs, pericytes, fibroblasts, osteoblasts, osteoclasts, macrophages, mast cells, and mesenchymal stem cells (MSCs) (Kopp et al. 2005). These cells constitute specialized niches, closed to the endosteum, named "osteoblast or endosteal niche", or to the BM vasculature, named vascular niche (Wilson and Trumpp 2006) (Fig. 1.3).

Bissell and Radisky (2001) pointed out that tumors evolve from an organ and retain memory of that organ and that once they liberate themselves from the con-

straints of the normal tissue microenvironment and lose the organ-specific structure as they evolve into the tumor organs and constantly redefine themselves by their ever-changing context.

1 Metastatic Cascade

In 1899, Stepen Paget proposed in a study conducted on autopsy records of 735 women who had died of breast cancer, that the process of metastasis did not occur by chance but, rather, that certain favored tumor cells, with metastatic activity (the "seed") had a special affinity for the growth-enhancing milieu within specific organs (the "soil"), i.e. organs providing a growth advantage to the seeds. He concluded that metastases developed only when the seed and soil were compatible (Paget 1899) (Fig. 1.4). In other words, Paget suggested that the site of metastasis depended on the affinity of the tumor to the microenvironment and this theory has been widely accepted as a basic principle in the field of cancer metastasis.

In 1928, James Ewing proposed that metastatic dissemination of cancer was dependent on the anatomical distribution of the vascular system (Ewing 1928) (Fig. 1.5). This controversy was resolved by Fidler (Fig. 1.6) and collaborators, who studied experimental metastasis in syngeneic mice to show that subsequent metastatic growth at a distant organ site was site-specific, consistent with Paget's original hypothesis (Hart 1979).

Fig. 1.4 A portrait of Stephen Paget. In 1899, he published his "seed and soil" explanation of non-random pattern of metastasis (Reproduced from Ribatti (2010))

Fig. 1.5 A portrait of James Ewing. In 1928, he proposed that metastasis occurs purely by anatomic and mechanical routes (Reproduced from http://wilkipedia.org)

Fig. 1.6 A portrait of Isaiah Fidler (Reproduced from http://www.mdanderson.org)

It has long been accepted that most malignant tumors show an organ-specific pattern of metastasis. For example, colon carcinomas metastasize usually to liver and lung but rarely to bone, skin or brain and almost never to kidney, intestine or muscle. In contrast, other tumor entities, such as breast carcinomas, frequently form metastases in most of these organs.

This specific formation of secondary tumors at distant sites appears to require a number of steps which must be successfully completed by metastasizing tumor cells (Chambers et al. 2002). Various explanations have been proposed for the site selectivity of blood-bone metastases, including tumor cell surface characteristics (Turner 1982), response to organ-derived chemotactic factors (Hujanen and Terranova 1985), adhesion between tumor cells and the target organ components (Nicolson 1988) and response to specific host tissue growth factors (Nicolson and Dulski 1986). The relative importance of pre-existing tumor subpopulations with specific metastatic properties and the organ environment characteristics in determining metastatic homing have been debated (Weiss 1979; Talmadge and Fidler 1982).

An alternative explanation for the different sites of tumor growth involves interactions between the metastatic cells and the organ environment, in terms of specific binding to ECs and responses to local growth factors. The mechanisms by which cancer cells transmigrate through the endothelial lining are not well understood. Signaling cross-talk between cancer cells and ECs may involve upregulation of adhesion molecule expression by the endothelium as well as by the tumor cells, reorganization of the acto-myosin cytoskeleton, and Src-mediated disruption of endothelial VE-cadherin-β-catenin cell-cell adhesion.

ECs in the vasculature of different organs express different cell-surface receptors and growth factors that influence the phenotype of the corresponding metastases. Greene and Harvey (1964) suggested that the organ distribution patterns of metastatic foci were dependent on the formation of sufficient adhesive bonds between arrested tumor cells and ECs, and they hypothesized that these interactions were similar to lymphocyte/ECs at sites of inflammation. The development of organ-derived microvascular EC cultures has allowed more specific studies on the preferential homing of tumor cells. Auerbach et al. (1985, 1987) found that different tumors showed differences in their adhesive propensity and preference for different ECs, and in a few cases preferential adhesion was observed to the ECs derived from the organ of origin and the target organ.

In a number of cancer types, including breast cancer, melanoma, prostate cancer, gastric cancer, and colon cancer, the occurrence of metastasis in the tumor draining lymph node (the "sentinel" lymph node) is regarded the first step in metastatic cancer dissemination. Tumor-induced changes in the sentinel lymph node include changes in the immune composition of the lymph node, in terms of changes in the density and maturation state of dendritic cells and T cells, in the cytokine composition, and the ability of the immune cells to generate a specific immune response (Cochran et al. 2006).

Before metastatic dissemination, primary tumors secrete factors that contribute to the development of a pre-metastatic niche, characterized by the presence of an increased number of fibroblasts and secreted cytokines rendering the microenvironment receptive to tumor growth. Bone marrow-derived VEGFR1$^+$ cells interfered with the formation of these clusters and blocked metastasis (Kaplan et al. 2005). At secondary sites, angiogenic dormancy is characterized by avascular micrometastatic lesions that do not grow beyond 1–2 mm in diameter because of balance

between proliferation and apoptosis. A dormant niche is associated with mature vessels and endothelial derived thrombospondin-1 (TSP-1), whereas metastatic outgrowth is associated with sprouting vessels and production of periostin and transforming growth factor beta (TGF-β) (Ghajar et al. 2013).

2 Epithelial-Mesenchymal Transition

One of the initial steps of primary tumor invasion is the epithelial mesenchymal transition (EMT), during which tumor cells lose epithelial markers and gain mesenchymal traits that confer stem-like properties and a migratory phenotype (Mani et al. 2008) (Fig. 1.7). During EMT, epithelial cells exhibit morphological changes: tight junctions, adherens junctions, desmosomes, and gap junctions are lost and the cells undergo actin cytoskeleton reorganization. Decreased E-cadherin function is another component of EMT, invasive tumor growth and metastasis. Perl et al. (1998), using E-cadherin knock-out mice and dominant-negative forms of this protein, showed that loss of E-cadherin was associated with pancreatic cell carcinogenesis.

The EMT promotes the generation of tumor associated fibroblasts (TAFs) whereby tumor cells of epithelial origin dedifferentiate to generate a mesenchymal-like population that express TAF markers (Petersen et al. 2003). At later stages of metastasis, secondary lesions display an epithelial-like phenotype, suggesting that mesenchymal-epithelial transition (MET) is important for metastatic outgrowth (Chaffer et al. 2007). TGF-β promotes EMT and single cell motility, which enables invasion into blood vessels, while in the absence of TGF-β cells are restricted to

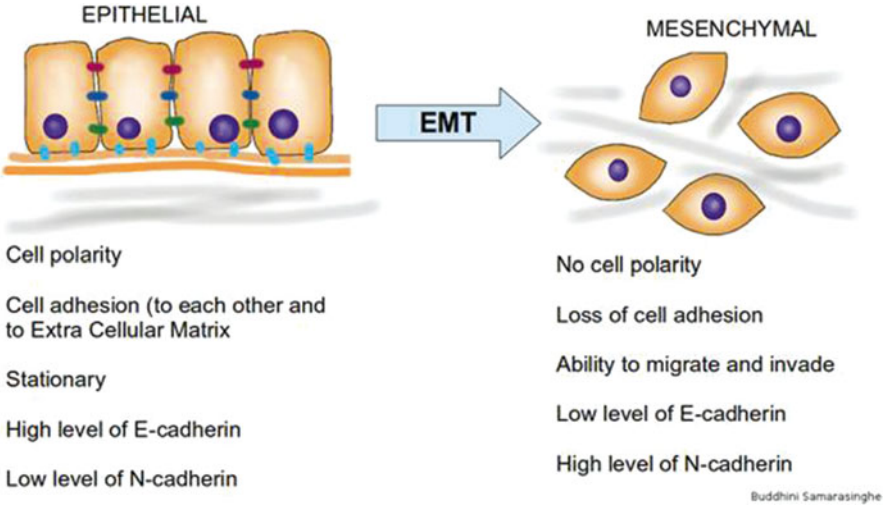

Fig. 1.7 Schema of epithelial-mesenchymal transition (Reproduced from Samarasinghe (2013))

collective movement and lymphatic spread (Giampieri et al. 2010). TGF-β is an early tumor suppressor that can subsequently promote tumor progression through tumor-stroma interactions resulting in metastasis, immune evasion, and stimulation of angiogenesis (Ikushima and Miyazono 2010).

Macrophages, platelets and MSCs contribute to EMT at primary sites, allowing tumor cells to separate from neighboring epithelial cell-cell contacts and acquire a mobile and invasive phenotype. During intravasation of tumor cells into the circulation, intravital imaging studies have shown that macrophages are localized to perivascular areas within tumors, where they help tumor cells to traverse vessel barriers. Platelets escort tumor cells in the circulation to the site of extravasation, where they bind to areas of vascular retraction and help tumor cells to exit the circulation into secondary organs. Myeloid derived suppressor cells (MDSCs) and natural killer (NK) cells populate pre-metastatic niches, where they help metastatic dissemination by creating a niche permissive to tumor colonization. Fibroblasts upregulate fibronectin, which serves as docking for HSCs and the arrival of tumor cells.

3 The Extracellular Matrix

The extracellular matrix (ECM) is composed of a biopolymer fiber networks of proteins, proteoglycans that differ in composition and structure between different locations. The ECM forms a mechanically stable support for epithelial cells, allows for the diffusion of oxygen and nutrients between the microvasculature and adjacent tissues and enables the trafficking of freely moving cells (Fig. 1.8).

The ECM provides critical and biomechanical cues that direct cell growth, survival, migration and differentiation, and modulate vascular development and immune function. Diseases associated with biochemical ECM changes are correlated with increased propensity for the development of cancer. A collagen-rich ECM promotes macrophage proliferation and activation (Welsey et al. 1998) and favors a

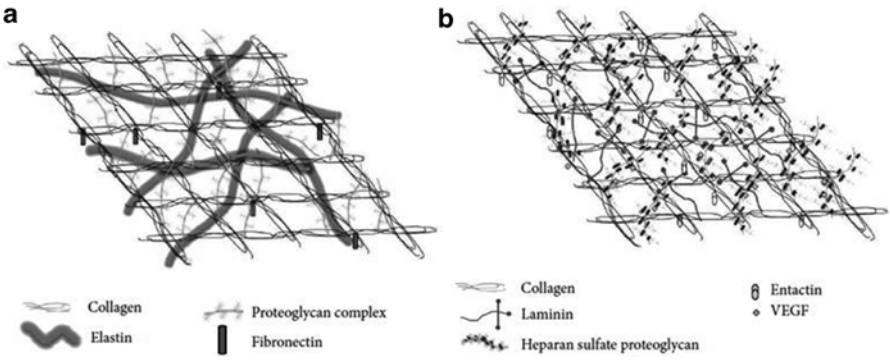

Fig. 1.8 A schematic drawing of the extracellular matrix organization (Reproduced from Neve et al. (2014))

pro-tumorigenic M2 polarization phenotype, whereas a fibronectin-rich ECM promotes the M1 or anti-tumorigenic potential of macrophages (Perri et al. 1982; Sathl et al. 2013).

A correlation between fibrotic stroma and cancer is well established in the liver (Maher and Bissell 1993) and increased stromal density correlates with a higher likelihood of developing breast cancer (Wolfe 1976). Gene-expressing profiling has shown that genes encoding for ECM components are dysregulated during tumor progression (Eckhardt et al. 2005).

Chapter 2
Tumor Blood Vessels and Tumor Endothelial Cells

Abstract Tumor endothelial cells contribute to tumor progression through recruitment of endothelial precursor cells and changes in the structure of the existing vascular endothelium creating an escape route for cancer cells to generate metastases. The tumor-associated endothelium is structurally defective, express several cell surface markers absent in quiescent blood vessels, and present several gene expression abnormalities. Pericytes deficiency could be responsible for vessel abnormalities in tumor blood vessels and partial dissociation of pericytes contribute to increased tumor vascular permeability.

Keywords Angiopoietin • Basement membrane • Capillary • Endostatin • Gene expression profile • Laminin • Multiple myeloma • Neuroblastoma • Pericyte • Platelet derived growth factor • Tenascin • Tumor blood vessels • Vascular endothelial growth factor • Vascular endothelial growth factor receptor • Vascular leakage

Tumor blood vessels are irregular in size, shape, and branching pattern, lack the normal hierarchy and do not display the recognizable features of arterioles, capillaries or venules. Their haphazard branching patterns and larger, less regular diameters contribute to the non-uniform perfusion of tumor cells. Tumor vessel leakiness and compression create a vicious cycle responsible for both acute and chronic hypoxia.

Abnormalities are present in all components of the vessels wall. The organ and tissue-specific vascular architecture is not retained. Even large caliber vessels are mainly composed of just an endothelium and a basement membrane.

Vessel density is heterogeneous: the highest values are usually found in what is commonly known as the invading tumor edge, where the density is four to ten times greater than inside the tumor (Giatromanolaki et al. 2002). The arrangement of vessels in the centre of a tumor is much more chaotic than at its edges.

ECs of mature, quiescent vessels are characteristically low proliferative and their estimated turnover times are measured in years, whereas those of tumor vessels are markedly dependent on growth factors for survival. VEGF has been assigned a central role in the induction of host vessels into a growing tumor.

ECs contribute at least in two ways to tumor progression; first, recruitment of EPCs stimulates the sprouting of new tumor blood vessels; second, changes in the structure of the existing vascular endothelium create an escape route for cancer cells to generate distant metastases.

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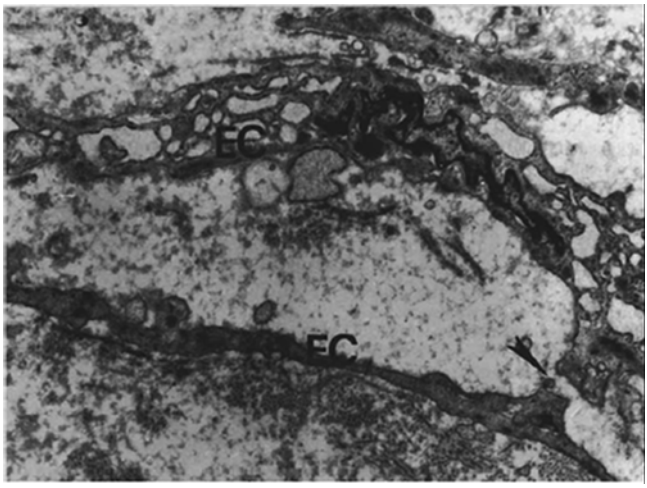

Fig. 2.1 Ultrastructural morphology of a fenestrated capillary in the stroma of a low-grade human lymphoma showing flat endothelial cells (EC) with a fenestra (*arrowhead*) (Reproduced from Ribatti et al. 1996)

When ECs invade a newly formed tumor, they come into contact with tumor cells that produce VEGF, which may be responsible not only for vascular proliferation, but also for the altered permeability of the newly formed vessels.

Tumor ECs proliferate 50–200 times faster than normal EC (Vermeulen et al. 1995). Their constantly proliferation rate reflects the angiogenesis that accompanies an increase in tumor volume, whereas in other regions they undergo apoptosis in parallel with tumor necrosis and vessel regression.

The tumor-associated endothelium is structurally defective. Discontinuities or gaps (<2 μm in diameter) that allow hemorrhage and facilitate permeability are common features (Fig. 2.1). Cells contacts are usually poorly differentiated and no complex contact structures exist. Defects in EC barrier function, due to abnormal cell-cell junctions and other changes, exaggerate leakiness. This correlates with histological grade and malignant potential (Daldrup et al. 1998) and can be exploited in locating tumors by imaging contrast media and in the delivery of macromolecular therapeutics (Mc Donald and Choyke 2003). Furthermore, it results in extravasation of plasma proteins and even erythrocytes and may facilitate the traffic of tumor cells into the bloodstream and the formation of metastases (Dvorak et al. 1988). Leakiness has been attributed to highly active angiogenesis and microvascular remodeling, but its structural basis and mechanism are unclear. Intercellular gaps, transendothelial holes, vesciculo-vacuolar organelles (VVOs, traverse ECs from lumen to albumen and additionally open to the interendothelial cell cleft), and endothelial fenestrae are present in the endothelium of tumor vessels (Dvorak et al. 1988).

Tumor ECs also differ for the profile and level of cell adhesion molecule they express. They preferentially overexpress the cell-surface molecules that are absent in quiescent blood vessels, including integrin αvβ3 and αvβ5, the fibronectin EBD domain, E-selectin, endoglin, endosialin, VEGFRs, and prostate specific membrane

Table 2.1 Inhibition of integrin signaling represses invasion and integrin downstream represent viable therapeutic targets for anticancer treatment

Inhibitors	Target integrin
Vitaxin-Medi 522	αvβ3
Cilengitide-EMD 121974	αvβ3, αvβ5
CNTO 95	αv
ATN-162	αvβ3, α5β1
E7820	α2 subunit
VOLOCIXIMAB, EOS-200-4, M200	α5β1

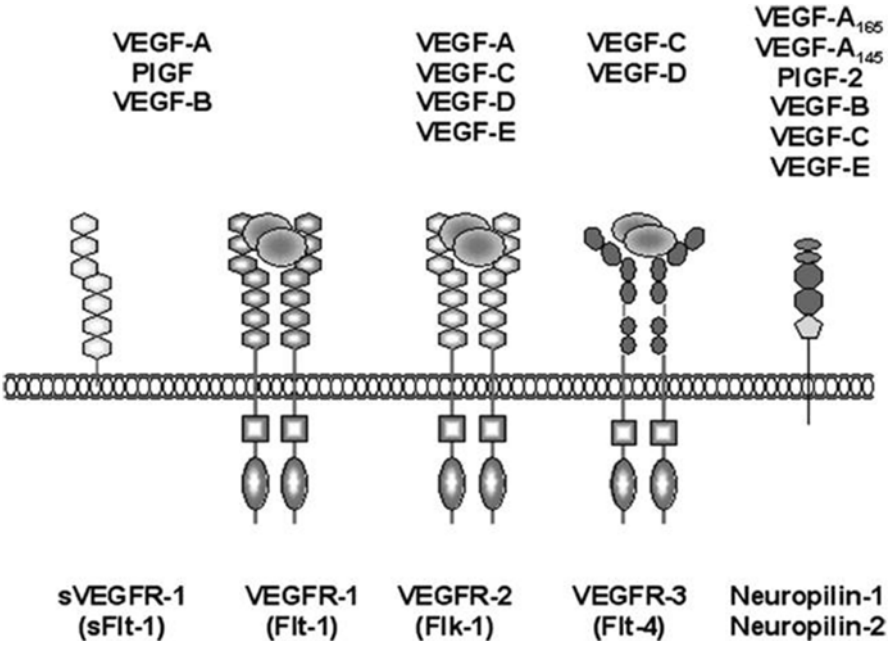

Fig. 2.2 VEGF growth factor and VEGF receptors (Reproduced from Ribatti 2005)

antigen (PSMA), all of which stimulate endothelium adhesion and migration (Thorpe 2004; Magnussen et al. 2005). Tumors treated with antagonists of integrin αv β3 display increased apopotosis and vessel regression (Table 2.1). One feature of the role of integrin αv β3 is that it mediates ECs adhesion to ECM molecules. Vascular endothelial cadherin (VE-cadherin) is poorly expressed in tumor vessels. It results in their destabilization and may lead to abnormal remodeling.

ECs lining tumor blood vessels express several cell surface markers that are absent in quiescent blood vessels. Ligand-directed vascular targeting can be accomplished by antibodies, specific peptides or growth factors complexed with immunomodulatory, procoagulant or cytotoxic molecules (Thorpe 2004).

VEGF and its receptors (Fig. 2.2) are up-regulated in tumor microenvironment, leading to a high concentration of occupied receptors on tumor vascular endothelium. VEGF immediate, short term (minutes) activity consists in increase of micro-

vascular permeability to plasma and plasma proteins with a potency some 50,000 times that of histamine (Senger et al. 1986). In the middle to longer terms (days, weeks) VEGF induces EC proliferation and survival (Ribatti 2005).

VEGFRs complexes are specific target on tumor endothelium for antibodies in vivo. Targeting VEGF or its receptors with monoclonal antibodies (such as bevacizumab) or small molecule inhibitors of VEGFR tyrosine kinase (such as ZD6476 and PTK787) has confirmed the anticancer activity of these agents (Ferrara 2002). Fusion proteins and chemical conjugates of VEGF and diphtheria toxin or gelonin induced tumor regression in mice (Thorpe 2004). Retroviruses have been engineered so that they can be coated with an antibody (e.g., anti-VEGFR-2) for the selectively delivery of genes to tumor endothelium (Masood et al. 2001).

The phage display library technique has been successfully used to discover tumor cell surface binding peptides. A panel of peptide motifs, including the sequences CDCRGDCFC (termed RGD-4C), NGR, CPRECES and GSL, have been assembled that target the tumor blood vessels (Kolonin et al. 2001).

ECs of arteries, capillaries, and veins exhibit differential gene expression and molecular identities already prior the onset of blood flow (Langenkamp and Molema 2009), indicating the importance of hitherto unidentified microenvironmental factors.

Tumor ECs present many gene expression abnormalities. St Croix et al. (2000) found that tumor-activated endothelium overexpressed specific transcripts as the result of qualitative differences in gene profiling compared with the normal ECs of the tissue of origin. A total of 79 transcripts were differentially expressed: 46 were elevated at least tenfold and 33 were expressed at substantially lower levels in tumor-associated ECs. Ria et al. (2009) have identified gene differentially expressed in multiple myeloma (MM) ECs as compared to monoclonal gammopathy of undertermined significance (MGUS) ECs. De-regulated genes were mostly involved in ECM formation and bone remodeling, cell adhesion, chemotaxis, angiogenesis, resistance to apoptosis, and cell-cycle regulation (Fig. 2.3).

Tenascin-C positive human neuroblastoma cells transdifferentiate into tumor-derived ECs which have been detected both in primary tumors and in tumors formed by human neuroblastoma cell lines in immunodeficient mice (Pezzolo et al. 2011). Tumor-derived ECs targeted with cytotoxic human CD31 monoclonal antibody enhance tumor hypoxia, which in turn induces EMT and endothelial transdifferentiation of tumor cells (Pezzolo et al. 2014).

1 Tumor Basement Membrane

The basement membrane that envelops ECs and pericytes (PCs) of tumor vessels may have extra layers that have no apparent association with the cells (Baluk et al. 2003). It contains distinctive forms of fibronectin comprising the ED-B domain, laminin and type IV collagen with exposed cryptic sites (Santamaria et al. 2003), and proteoglycans that limit the expansion of normal microvessels to about 30 % (Chang et al. 2009). Fibronectin, laminin, collagen I, III, and IV play distinct and overlapping roles in angiogenesis.

Gene	Chromosoma	Protein	Function
BNIP 3	10q26.3	BCL2/adenovirus 19kDa interacting protein 3	Trascriptional factor regulated by hypoxia
IER 3	6p21.3	Immediate early response 3	Anti-apoptosis
SEPW1	19q13.3	Selenoprotein W, 1	omeostasys
CRYAB	11q22.3-q23.1	Crystallin, alpha B	Tubular morphogenesis
SERPINF1	17p13.1	Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	Anti-angiogenesis
SRPX	Xp21.1	Sushi-repeat-containing protein, X-linked	Oncosuppressor

Fig. 2.3 Genes differentially expressed in multiple myeloma endothelial cells (MMECs) as compared to endothelial cells of monoclonal gammopathy of undetermined significance (MGECs) (Reproduced from Ribatti and Vacca 2013)

ECM is a source of angiogenic and antiangiogenic molecules. Some of its structural proteins are broken down by enzymes to yield molecules with potent actions. Basement membrane degradation results from the increased expression and activation of pericyte cathepsin proteases, accompanied by decreased expression of cysteine protease inhibitors that limit cathepsin activity (Chang et al. 2009).

Three examples of anti-angiogenic molecules are endostatin, which is a COOH-terminal fragment of collagen XVIII, tumstatin and arresten, which are the noncollageneous-1 domain of the α3 and the α1 chains of type IV collagen respectively (Colorado et al. 2000). Laminin, which consists of heterodimeric α, β, and γ chains, has been implicated in invasion and metastasis in various type of cancers (Akimoto et al. 2004). An anti-laminin peptide inhibits bone metastases of the A375 human melanoma cells and the MH-95 B-cell lymphoma cells (Nakai et al. 1992; Michigami et al. 1998). Basement membrane is also a site of growth factor binding and a participant in angiogenesis (Kalluri 2003).

2 Tumor Pericytes

PCs have been characterized by the expression of alpha-smooth actin (α-SMA), desmin, CD146, platelet derived growth factor receptor beta (PDGFRβ), and nerve/glial antigen 2 (NG2) proteoglycan (Ribatti et al. 2011).

Birbrair et al. (2013) identified two PCs subpopulations from large blood vessels and capillaries, named type-1 and type-2 PCs. *In vitro* and in vivo assays revealed that type-2 (Nestin-GFP$^+$/NG2-DsRed$^+$), but not type-1 (Nestin-GFP$^-$/NG2-DsRed$^-$) PCs are recruited during tumor angiogenesis (Ribeiro and Okamoto 2015). In tumors, NG2 is a marker of a mature population of PCs (Song et al. 2005). Desmin positivity represents a more mature population of PCs (Song et al. 2005). In tumors, desmin-positive PCs appear more closely associated with vessels, whereas αSMA-positive and PDGFR-β-positive are partially detached (Morikawa et al. 2002).

PCs may cover 73–92 % of endothelial sprouts in several murine tumor types. PCs are located near blood vessels at the growing front of tumors, where angiogenesis is most active and show morphological abnormalities (Schlingemann et al. 1990; Wesseling et al. 1995; Morikawa et al. 2002). Breast and colon tumors recruit significantly more PCs than gliomas or renal cell carcinoma (Eberhard et al. 2000).

PC deficiency could be partly responsible for vessel abnormalities in tumor blood vessels (Gerhardt and Semb 2008) and partial dissociation of PCs (Hobbs et al. 1998; Hashizume et al. 2000) contribute to increased tumor vascular permeability.

Severe reduction or lack of PC coverage may disrupt the integrity of the vasculature, enabling tumor cells to transit into the circulatory system, thereby facilitating metastasis. Genetic disruption of PC coverage elicited increased metastasis in the Rip1-Tag2 pancreatic islet tumor model (Xian et al. 2006). Moreover, the increased interstitial pressure compress other tumor vessels, triggering tumor metastasis through a hypoxia-induced EMT mechanism (Cooke et al. 2012).

Tumor PCs are loosely associated with ECs, have abnormal shape, paradoxically extend cytoplasmic processes away from the vessel wall, and have extra layers of loosely fitting basement membrane (Eberhard et al. 2000). Their abnormalities are consistent with alterations in PDGF signaling pathways. Mice genetically deficient in PDGFB or its receptors have blood vessels with loose PC attachment, irregular vessel caliber, luminal projections on ECs, and hemorrhage. Similar abnormalities occur in many tumor vessels. PDGFB expressed by tumor cells increases PC recruitment in several in vivo models (Guo et al. 1985). Alternatively, genetic abolition of PDGFB-R expressed by embryonic PCs decreased their recruitment (Abramsson et al. 2003). In Lewis lung carcinoma implanted in mice, inhibition of endothelial differentiation gene-1 (EDG-1) expression in ECs strongly reduced PC coverage (Chae et al. 2004). In a human glioma model developed in rat, angiopoietin-1 (Ang-1) led to enhanced PC recruitment and increased tumor growth, presumably by favoring angiogenesis (Machein et al. 2004). Alternatively, in a colon cancer model, overexpression of Ang-1 led to smaller tumors with fewer blood vessels and greater PC coverage, decreased vascular permeability and reduced hepatic metastasis (Ahmad et al. 2001).

In a human neuroblastoma xenotransplanted model, PC coverage is halved in tumors grafted on matrix metalloproteinase-9 (MMP-9)-deficient mice and transplantation with MMP-9-expressing BM cells restores the formation of mature vessels (Chantrain et al. 2004). In addition, overexpression of tissue inhibitor of MMP-3

Table 2.2 Pericyte function can be targeted by kinase inhibitors modulating PDGFRβ signaling

Sunitinib malate
CP-673451
Sorafenib tosylate
Orantinib (TSU-68)
MK-2461
Linifanib
Axitinib
Imatinib
Crenolanib
Dovitinib
Nintendanib

(TIMP-3), results in decreased PC recruitment in neuroblastoma and melanoma models (Spurbeck et al. 2002). The observation that PCs express MMPs in many human tumors in vivo (Nielsen et al. 1997) suggests that PC invasion requires the proteolytic degradation of extracellular matrix by proteases, including MMPs.

VEGF inhibition eliminates tumor vessels without removing PCs (Morikawa et al. 2002). Anti-angiogenic treatment directed against ECs using VEGF inhibitors induces the regression of tumor vessels and decreased tumor size (Baluk et al. 2005), leading to vessel normalization, characterized by increased PC coverage, tumor perfusion and chemotherapeutic sensitivity (Jain 2005). Moreover, removal of VEGF inhibition causes tumor re-growth due to the fact that PCs provide a scaffold for the rapidly re-growing of tumor vessels (Mancuso et al. 2006).

PCs have been indicated as putative targets in the pharmacological therapy of tumors by using the synergistic effect of anti-endothelial and anti-pericytic molecules (Table 2.2). Removal of PC coverage leads to exposed tumor vessels, which may explain the enhanced effect of combining inhibitors that target both tumor vessels and PCs. Bergers et al. (2003) showed that combined treatment or pre-treatment with anti-PDGF-B/PDGFBR-β reducing PC coverage increases the success of anti-VEGF treatment in the mouse RIP1-TAG2 model.

However, extensive regression of ECs was not observed in tumors after inhibition of PDGFR-β signaling (Abramsson et al. 2003). STI571 (Gleevec, Imatinib), which targets PDGFRs and other receptor tyrosine kinases, did not reduce vascular density when given alone but did augment the effects of VEGF inhibitors (Bergers et al. 2003). After treatment of RIP1-TAG-2 tumors and Lewis lung carcinomas with AG-013737 or VEGF-Trap, surviving PCs may become more tightly associated with ECs or have no apparent association with tumor vessels (Inai et al. 2004). Treatment of RIP1-TAG2 tumors with anti-PDGFR-β antibody for 3 weeks reduces PCs, increases EC apoptosis but does not seem to reduce tumor vascular density (Song et al. 2005). Similarly, the receptor tyrosine kinase inhibitor SU6668, which also affects PDGFR-β signaling, detaches and reduces PCs in RIP1TAG2 and xeno-transplanted tumors, thereby restricting tumor growth (Reinmuth et al. 2001;

Shaheen et al. 2001). Sennino et al. (2007) demonstrated that treatment with a novel DNA oligonucleotide aptamer (AX102) that selectively binds PDGF-B led to progressive reduction of PCs in Lewis lung carcinomas. Murphy et al. (2010) generated a series of selective type II inhibitors of PDGFR-β and B-RAF targets for PC recruitment and endothelial survival, respectively, and demonstrated that dual inhibition of both PDGFR-β and B-RAF exerted synergistic anti-angiogenic activity in both zebrafish and murine models of angiogenesis.

Chapter 3
Tumor Angiogenesis

Abstract Tumor angiogenesis may occur through sprouting angiogenesis; recruitment of bone marrow-derived endothelial precursors cells (postnatal vasculogenesis); vasculogenic mimicry; mosaic vessel formation intussusceptive microvascular growth (IMG).

Keywords Angiogenesis • Anti-angiogenesis • Chorioallantoic membrane • Glioblastoma multiforme • Hepatocellular carcinoma • Intussusceptive micorvascular growth • Lymphoma • Mammary carcinoma • Matrix metalloproteinases • Melanoma • Sirolimus • Sprouting angiogenesis • Tumor angiogenesis factor • Tyrosine kinase inhibitors • Vascular cooption • Vasculogenic mimicry

In 1971, Judah Folkman (Fig. 3.1) published his classic article in which he formulated three bold postulates: (i) angiogenesis is essential for tumor growth beyond minimal size; (ii) tumors secrete a "tumor angiogenesis factor" (TAF) responsible for inducing angiogenesis; (iii) anti-angiogenesis is a potential therapeutic strategy for treating cancer.

Within the decade immediately following Folkman's paper the field of angiogenesis grew exponentially and over 30 years after Folkman proposed the concept of the anti-angiogenic treatment of cancer, the idea finally became clinical practice. In the meantime, Folkman proposed the concept of the so-called angiogenic switch, which could be defined as the conversion from a dormant to an activated state (Ribatti et al. 2007). When this balance is disturbed and pro-angiogenic factors are in excess, rapid vascularization and growth of tumors may occur.

Tumor angiogenesis may occur through several mechanisms: (i) sprouting angiogenesis; (ii) recruitment of bone marrow-derived EPCs to form new vessels (postnatal vasculogenesis) (Ribatti 2007); (iii) vasculogenic mimicry (the transdifferentiation of cancer cells allowing them to form tubular structures themselves) (Maniotis et al. 1999); (iv) mosaic vessel formation (the incorporation of cancer cells into the vessel wall or vascular cooption) (Holash et al. 1999); (v) intussusceptive microvascular growth (IMG) (Ribatti and Djonov 2012).

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Fig. 3.1 A portrait of Judah Folkman, a pioner in the study of angiogenesis (Reproduced from Ribatti (2010)

1 Sprouting Angiogenesis

Sprouting angiogenesis corresponds to the process through which new vessels extend from pre-existing vasculature by formation of a vascular sprout. ECs acquire the capacity to invade the surrounding tissue by forming an angiogenic sprout composed of a leading tip cell responsive a to angiogenic stimuli and trailing stalk cells that function in tube formation which orientate toward the source of the angiogenic stimulus (Ribatti and Crivellato 2012b). The progression of angiogenesis is initiated by local destruction of the BM of a vessel and the dissociation of PC from the capillary, followed by migration of tip cells toward the angiogenic stimulus. Over time a lumen is formed and once the sprout anastomoses with another vessel sprout, circulation is established. PC and/or smooth muscle cell association and basement membrane deposition mediate vessel stabilization.

2 Vascular Co-option

Holash et al. (1999) reported that tumor cells migrate to host organ blood vessels in sites of metastases, or in vascularized organs such as the brain, and initiate blood-vessel-dependent tumor growth as opposed to classic angiogenesis, co-opting and growing as cuffs around adjacent vessels. These vessels then regress owing to apoptosis of the constituent EC, apparently mediated by Ang-2, resulting in a massive tumor death. Lastly, at the periphery of the growing tumor mass angiogenesis occurs by cooperative interaction of VEGF and Ang-2. Shortly after regression,

tumor upregulates its expression of VEGF, presumably because it is becoming hypoxic due to the loss of vascular support. As in normal vascular remodeling, the destabilizing signal provided by Ang-2, which leads to vessel regression in the absence of VEGF, potentiates the angiogenic response in combination with VEGF. Ang-2 induction in host vessels in the periphery of experimental C6 glioma precedes VEGF upregulation on tumor cells, and causes regression of co-opted vessels (Holash et al. 1999).

Vajkoczy et al. (2002) have demonstrated parallel induction of Ang-2 and VEGFR-2 in quiescent host ECs, suggesting that their simultaneous expression is critical for the induction of angiogenesis during vascular initiation of microtumors. The VEGF/Ang-2 balance may determine whether the new tumor vessels continue to expand when the ratio of VEGF to Ang-2 is high, or regress when it is low during their remodeling.

Vascular co-option is more frequently observed in cancer of densely vascularized organs, including brain, lung, liver, where the primary tumor cells co-opt the adjacent quiescent blood vessels of the host tissue.

3 Vasculogenic Mimicry

Maniotis et al. (1999) described a new model of formation of vascular channels by human melanoma cells and called it "vasculogenic mimicry" to emphasize the *de novo* generation of blood vessels without the participation of ECs and independent of angiogenesis. Microarray gene chip analysis of a highly aggressive compared with poorly aggressive human cutaneous melanoma cell lines revealed a significant increase in the expression of laminin 5 and MMP-1, MMP-2, MMP-9 and membrane type 1 (MT1)-MMP in the highly aggressive cells (Seftor et al. 2001), suggesting that increased expression of MMP-2 and MT1-MMP along with matrix deposition of laminin 5 are required for their mimicry. Another possibility is that the EC lining is replaced by tumor cells, resulting in "mosaic" vessels whose lumen is formed of both endothelial and tumor cells. Chang et al. (2000) used CD31 and CD105 to identify ECs and endogenous green fluorescent protein (GFP) labeling of tumor cells, and showed that approximately 15 % of perfused vessels of a colon carcinoma xenografted were mosaic, with focal regions where no CD31/CD105 immunoreactivity was detected and tumor cells were in contact with the vessel lumen.

We have demonstrated by using the chorioallantoic membrane (CAM) assay that the capability of B16-F10 melanoma cells to contribute to the new formation of host blood vessels through a vasculogenic mimicry mode Ribatti et al. 2013). Results have shown that B16-F10 melanoma cells are able to form in 4 days macroscopic tumor masses and induce a strong angiogenic response comparable to that of a well-known angiogenic cytokine, namely FGF-2. Moreover, tumor cells are able to cross the chorionic epithelium, and to move beneath in the mesenchyme to form tumor masses immunoreactive to specific antibodies anti-S100 and anti- MART-1/

Melan-A. Finally, we have shown that CAMs new-formed blood vessels are lined by both pigmented melanoma cells and cells immunoreactive to MART-1/Melan-A and PAS, suggesting the occurrence of a vasculogenic mimicry process (Fig. 3.2).

4 Intussusceptive Microvascular Growth

IMG generates vessels more rapidly with a less metabolic demand as compared to sprouting angiogenesis and is a putative strategy that tumors can use for rapid adaptation to milieu changes. In fact, no EC proliferation is required for IMG and ECs only increase their volume and become thinner (Fig. 3.3). IMG occurs in several tumors, including colon and mammary carcinomas, melanoma, B-cell non Hodgkin's lymphoma, and glioma (Patan et al. 1996; Djonov et al. 2001; Crivellato et al. 2003; Ribatti et al. 2005a; Nico et al. 2010). Patan et al. (1996) observed the growth of human colon adenocarcinoma *in vivo* and demonstrated that IMG is a mechanism in tumor angiogenesis, suggesting that the rapid vascular remodeling caused by IMG contributes to intermittent blood flow in tumors. Djonov et al. (2001) showed that in mammary tumors of neuT transgenic mice, both sprouting and IMG occur simultaneously in the same nodule. Crivellato et al. (2003) (Fig. 3.4) demonstrated that in B-cell non-Hodgkin's lymphomas, large vessels developed transluminal bridges leading to the division of the parental vessels into two or more parts. Moreover, different morphological structural changes in term of expression of IMG have been identified: pillar formation by folding of the lateral vascular wall, fusion of pillars and connection of intraluminal tissue folds with the opposite vascular wall, leading to the splitting of the original vascular structure into newly formed blood vessels (Crivellato et al. 2003). Ribatti et al. (2005a) and Nico et al. (2010) confirmed the presence of tumor vessels with connections of intraluminal tissue folds with the opposite vascular wall in human melanoma and, respectively, human glioma (Figs. 3.5 and 3.6).

Paku et al. (2011) by using electron and confocal microscopy, observed intraluminal nascent pillars that contain a collagen bundle covered by ECs and proposed a new mechanism for the development of pillars consisting of four steps: (i) formation of intraluminal endothelial bridges; (ii) on the abluminal side of the ECs that form the bridge the basement membrane is locally disrupted by proteolytic activity; (iii) an EC from the bridge adheres to a nearly collagen bundle which is transferred through the lumen, reaches the other side of the lumen, and is transferred into the connective tissue on the other side of the vessel; (iv) further pillar maturation occur through the immigration of fibroblasts/myofibroblasts and PCs into the pillar and subsequent ECM proteins (collagen and fibrin) deposition by these cells.

After initial tumor regression in response to an anti-angiogenic treatment, tumor may re-grow. The adaptive mechanisms by which tumor re-grow following development of resistance to anti-angiogenic drugs targeting mostly VEGF, include: (i) activation and/or up-regulation of alternative pro-angiogenic signaling pathways; (ii) recruitment of pro-angiogenic cells, including EPCs and bone marrow-derived

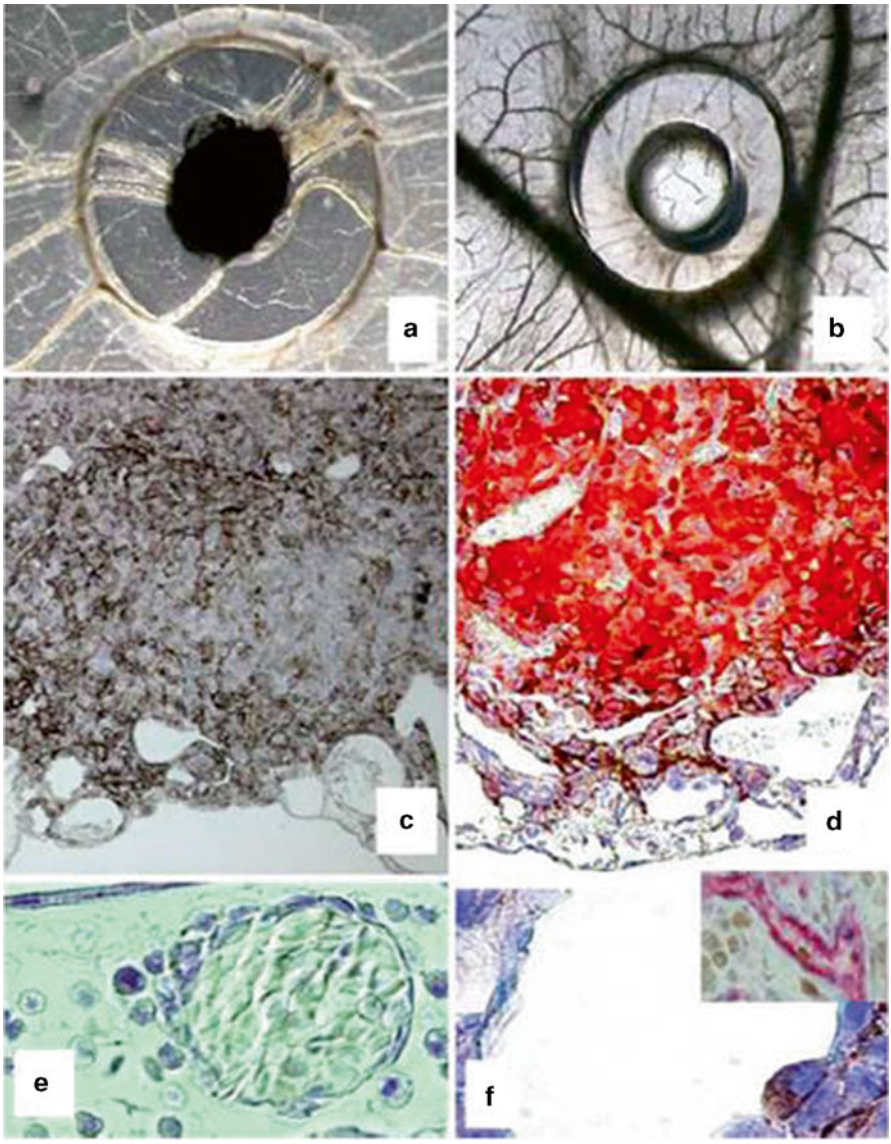

Fig. 3.2 Macroscopic pictures of CAMs at day 12 of incubation, showing silicon rings containing B16F10 melanoma cells suspensions. The cells give rise to distinct appreciable pigmented tumor masses inside (**a**) the silicon rings and are surrounded by numerous allantoic vessels. Few blood vessels are recognizable around a silicon ring treated with vehicle alone (**b**). In (**c**) and (**d**) microscopic pictures of CAMs at day 12 of incubation showing that B16F10 melanoma cells are densely packed inside the CAM mesenchyme are easily distinguishable as brown pigmented tumor masses (**c**) or reactive to anti-S100 specific antibody (**d**). In (**e**) and (**f**) microscopic pictures of CAMs at day 12 of incubation showing the morphological features of CAMs' blood vessels. They are lined by chick's endothelial cells (**e**), or melanoma cells showing a characteristic cytoplasmic immunoreactivity to the specific antibody anti-MART-1/Melan-A (**f**) or are positive to both MART-1/Melan-A/PAS (**f**, inset) (Reproduced from Ribatti et al. (2013))

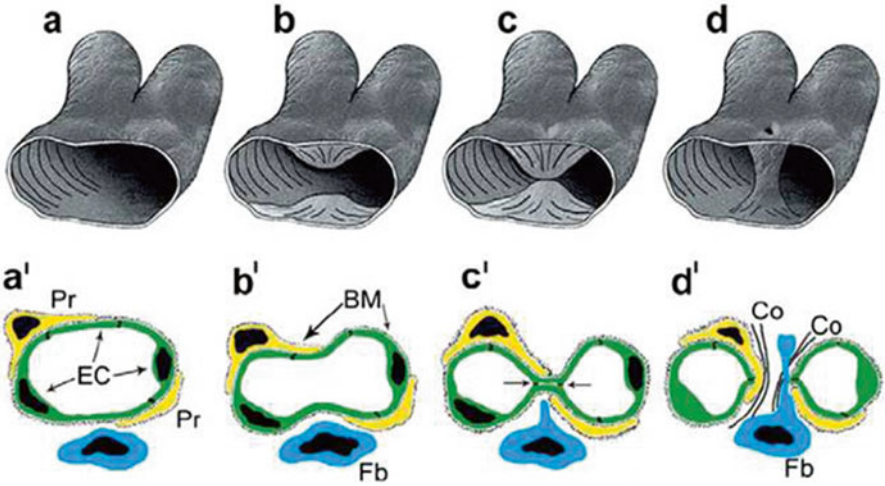

Fig. 3.3 3D (*a*–*d*) and 2D (*a′*–*d′*) scheme depicting the generation of transluminarly pillar by intussusceptive angiogenesis. Simultaneously protrusion of opposing capillary walls into the vessel lumen (*a*, *b*; *a′*, *b′*) results in creation of interendothelial contact zone (*c*; *c′*). In a subsequent step the endothelial bilayer becomes perforated and the newly formed pillar core got invaded by fibroblasts (*fb*) and pericytes (*Pr*), which lay down collagen fibrils (*Co* in *d′*) (Reproduced from Ribatti and Djonov (2012))

cells; (iii) increased pericyte coverage of the tumor vasculature to attenuate the necessity for VEGF-mediated survival signaling; (iv) activation and enhancement of invasion and metastasis; (v) activation of alternative mechanisms to form blood vessels, including IMG, co-option, and vasculogenic mimicry.

A switch from sprouting to intussusceptive angiogenesis might represent an adaptive response to treatment with various antitumor and anti-angiogenic compounds to restore the hemodynamic and structural properties of the vasculature enhancing tumor drug delivery and sensitivity to treatments.

In a hepatocellular carcinoma experimental model treated with sirolimus, a mTOR inhibitor, during the treatment and the early recovery phase vascular sprouting was absent, whereas IMG was observed (Semela et al. 2007). Whereas the capillary plexus expanded primarily by sprouting in the controls, this mechanism was nearly absent in the tumors of treated animals and replaced by intussusception (Semela et al. 2007).

Radiotherapy of mammary carcinoma allografts or treatment with an inhibitor of VEGF tyrosine kinase (PTK787/ZK222854) results in transient reduction in tumor growth rate with decreased tumor vascularization followed by post-therapy relapse with extensive IMG, characterized by a plexus composed of enlarged sinusoidal-like vessels containing multiple transluminal tissue pillars, a dramatic decrease in the intratumoral microvascular density, probably as a result of intussusceptive pruning associated to a minimal reduction of the total microvascular exchange area (Hlushchuk et al. 2008). Moreover, the switch to IMG improves the perfusion of the

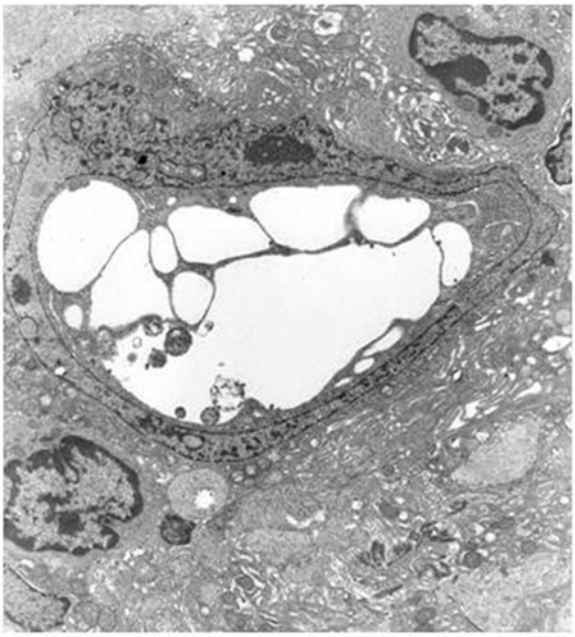

Fig. 3.4 Ultrastructural findings of human non-Hodgkin's lymphoma specimen. Transluminal bridges leading to the parental vessel in two or more parts are appreciable (Reproduced from Crivellato et al. (2003))

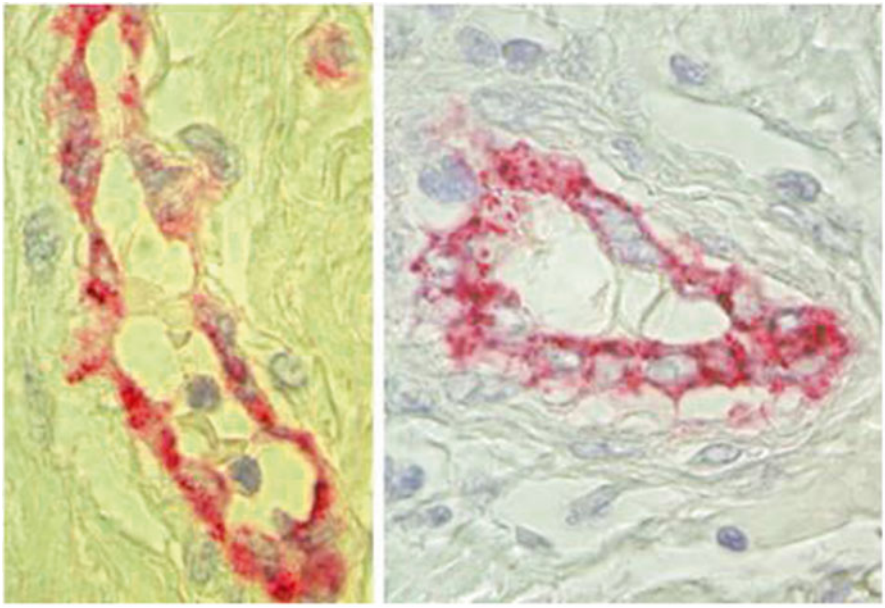

Fig. 3.5 Two examples of tumor vessels with a high and low number of intraluminal tissue folds with the opposite vascular wall, expression of intussusceptive microvascular growth in human primary melanoma tumor specimens (Reproduced from Ribatti et al. (2005a))

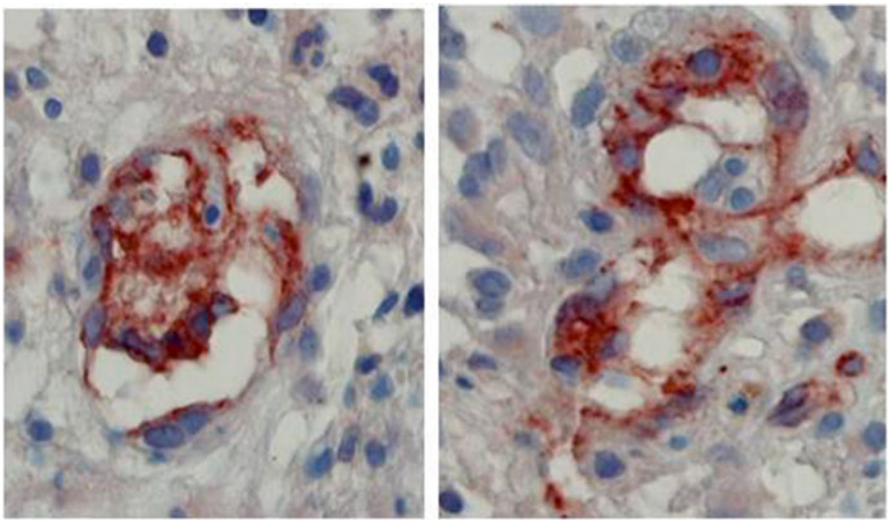

Fig. 3.6 Two examples of tumor vessels with a high and low number of intraluminal tissue folds with the opposite vascular wall, expression of intussusceptive microvascular growth in human glioma tumor specimens (Reproduced from Nico et al. (2010))

tumor mass as has been shown by an improvement in oxygen supply of the tumor mass (Hlushchuk et al. 2008).

Tumor recovery after treatment with inhibitors of VEGFR signaling in the RIP-Tag 2 and Lewis lung carcinoma models was associated with rapid revascularization and evidence of IMG (Mancuso et al. 2006). Similar changes in vasculature have been observed in a murine renal cell carcinoma study (Drevs et al. 2002), in murine orthotopic B16/BL6 melanoma tumor model after treatment with either PTK/ZK (Rudin et al. 2005) or other tyrosine kinase inhibitors (Nakamura et al. 2004, 2006; Ruggeri et al. 2003). Wnuk et al. (2011) demonstrated that restoration of glomerular capillary structure after induction of Thy1.1 nephritis occurred by intussusceptive angiogenesis and this in spite of the VEGFR-2 and PDGFRβ inhibition. The results provide an important insight into IMG control mechanisms and indicate differential molecular regulation between sprouting and intussusceptive angiogenesis.

Chapter 4
Inflammatory Cells in Tumor Microenvironment

Abstract Both innate and adaptive immune cells are involved in the mechanisms of tumor endothelial cell proliferation, migration and activation, through the production and release of a large spectrum of pro-angiogenic mediators, which create the specific microenvironment that favours tumor angiogenesis. Cancer stem cells have been identified in several human solid and hematological tumors and able to initiate tumor formation and metastasis. Cancer stem cells are more resistant to chemotherapeutic agents and radiation therapy than more mature cell types from the same tissue.

Keywords Cancer stem cells • Chromosomal genetic instability • Eosinophils • Dendritic cells • Fibroblasts • Granulocyte-colony stimulating factor • Granulocyte macrophage-colony stimulating factor • Inflammation • Interleukins • Lymphocytes • Macrophages • Mast cells • Multipotent adult progenitor cells • Myeloid-derived suppressor cells • Neutrophils • Osteoblasts • Osteoclasts • Platelets • Tie-2 expressing monocytes

Tumor cells undergo a Darwinian selection and are able to survive and enter into an equilibrium state in which the innate and adaptive immune system control the tumor (Dunn et al. 2002; Schreiber et al. 2011). Some tumor cells acquire mutations, chromosome amplifications and deletions, and epigenetic modifications, resulting in gene silencing or synthesis of abnormal proteins. Overall, these events allow tumor cells to escape the control of the immune system, proliferate and give rise to a clinically detectable tumor.

The historical observations by Paul Ehrlich (Fig. 4.1) that tumor cells are recognized and eliminated by the immune cells evolved in the theory of innate immune surveillance, proposed by Sir Marcfarlane Burnet (Fig. 4.2) in 1970. In this context, the immune system spontaneously identifies and eliminates cancer cells. Accordingly to the concept of immunoediting tumor cells that are vulnerable to attack by the immune system are cleared, whereas cells that have a capacity to circumvent immunosurveillance can survive. Immunodeficient mice are more prone to the development of spontaneous tumors, and carcinogen-induced sarcoma developed better in a host devoid of immunity (Shankaran et al. 2001).

The link between chronic inflammation and tumorigenesis was first proposed by Rudolf Virchow (Fig. 4.3) in 1863 after the observation that infiltrating leukocytes

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Fig. 4.1 A port trait of Paul Ehrlich (Reproduced from Ribatti (2010))

Fig. 4.2 A port trait of Sir Frank Macfarlane Burnet (Reproduced from Ribatti (2009))

Fig. 4.3 A port trait of Rudolf Virchow (Reproduced from http://wilkipedia.org)

are a hallmark of tumors and first established a causative connection between the lymphoreticular infiltrate at sites of chronic inflammation and the development of cancer (Balkwill and Mantovani 2002). Surgeons have long described the tendency of tumors to recur in healing resection margin and it has been reported that wound healing environment provides an opportunistic matrix for tumor growth (Abramovitch et al. 1998; Wong and Reiter 2011). Tumors were described by Dvorak as wounds that never heal (Fig. 4.4) (Dvorak 1986).

Population-based studies have shown that individuals who are prone to chronic inflammation disorders have an increased risk of cancer development (Table 4.1) (Balkwill et al. 2005). A meta analysis found that in ~15 % of cancers, tumor initiation can be attributed to infection by viruses, bacteria, and parasites (de Martel et al. 2012).

Chromosomal genetic instability in susceptible cell population can be induced by different factors such as reactive oxygen species released from inflammatory cells causing accelerated accumulation of genetic and epigenetic alterations that affect the expression or function of proto-oncogenes and tumor suppressor genes in somatic cells (Coussens and Werb 2002).

Treatment with non steroidal anti-inflammatory agents decreases the incidence and mortality of several tumor types and low dose aspirin could reduce the relative risk of cancer mortality (Koehne and Dubois 2004). Moreover, there is a strong evidence linking carcinogenesis to inflammatory response and reactive oxygen and nitrogen species, and therapeutic strategies for cancer prevention using free radicals and pro-inflammatory signaling inhibitors have been evaluated in animal models (Rao et al. 2002).

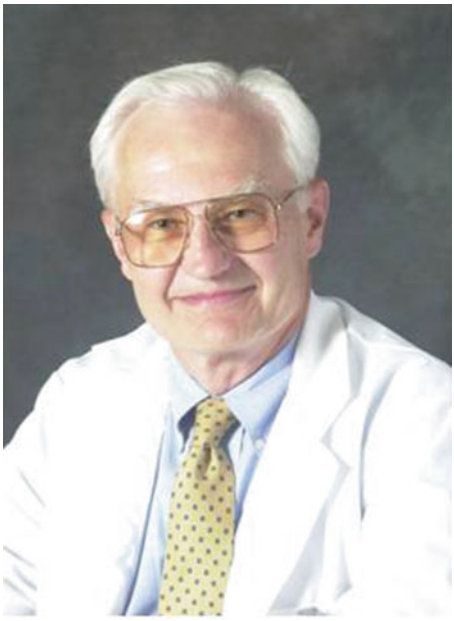

Fig. 4.4 A port trait of Harold Dvorak (Reproduced from Ribatti (2010))

Table 4.1 Different types of cancers are associated with chronic inflammatory disorders

Colorectal cancer, ulcerative colitis and Chron's disease
Cholangiocarcinoma, primary sclerosing cholangitis
Gastric cancer, chronic gastritis (*Helicobacter pylori*)
Lung cancer, inflammation caused by asbestsos, smoking, and sylica
Prostate cancer, *Escherichia coli* infection of prostate
Hepatocellular carcinoma, infection caused by hepatitis virus B and virus C
Melanoma, UV irradiation and associated skin inflammation
Endometrial carcinoma, endometriosis
Gallbladder carcinoma, gallbladder stone-associated chronic cholecystitis
Esophageal cancer, Barrett's esophagitis

The transition from a pre-malignant lesion to cancer is characterized by a shift of an immune context formed by Th1, Th17, T effector cells and M1 macrophages to a microenvironment where Th2 cells, Tregs, M2 macrophages and MDSCs are prevalent.

Under a variety of inflammatory conditions, both innate and adaptive immune cells are capable of polarization into their "tumoricidal" (growth arresting) or "tumorigenic" (growth promoting) forms.

1 Neutrophils

Neutrophils are the source of several cytokines, including TNF-α, inteleukin (IL-1β, IL-12), VEGF, and chemokines, after including change in chemokine (C-X-C motif) ligand (CXCL-1, 8,10), chemokine (C-C motif) ligand (CCL-3, 4), involved in angiogenesis (Scapini et al. 2000).

In breast cancer, release by tumor neutrophils of oncostatin M induce VEGF production from cancer cells (Queen et al. 2005). Expression of human papilloma virus (HPV) 16 in transgenic mice induces a multi-stage transformation of basal keratinocytes to squamous carcinoma, infiltration of neutrophils and mast cells, activated to secrete MMP-9, and angiogenic switch (Coussens and Werb 1996). In another experimental model of pancreatic carcinogenesis, Rip-Tag2, neutrophils express MMP-9 and their depletion reduced the angiogenic switch of premalignant lesions (Nozawa et al. 2006). Moreover, in knock-out mice for MMP-2 and MMP-9, the absence of both MMP-9-positive neutrophils and MMP-2-expressing-stromal cells resulted in the absence of tumor vascularization and invasion (Masson et al. 2005). Deryugina et al. (2014) demonstrated that tumor associated neutrophils are the major source of MMP-9 in the tumor microenvironment (Deryugina et al. 2014).

Expression of the hematopoietic cytokines granulocyte-colony stimulating factor (G-CSF) and/or granulocyte macrophage-colony stimulating factor (GM-CSF) was associated with neutrophil recruitment and angiogenesis in malignant HaCaT tumor cells (Obermueller et al. 2004; Gutschalk et al. 2006).

2 Eosinophils and Dendritic Cells

Eosinophils produce a variety of pro-angiogenic factors, including VEGF, CCL-11, fibroblast growth factor-2 (FGF-2), CXCL-8, GM-CSF, PDGF, TGF-β, and MMP-9 (Puxeddu et al. 2005; Salcedo et al. 2001; Munitz and Levi-Schaffer 2004; Ohno et al. 1997). Using in vitro and in vivo models, such as aorta ring and CAM assay, eosinophils induce angiogenesis (Puxeddu et al. 2005; Salcedo et al. 2001).

Dendritic cells may contribute to angiogenesis by secretion of pro-angiogenic factors (Table 4.2) or transdifferentiation into endothelial like cells. Riboldi et al. (2005) demonstrated that alternatively activated dendritic cells produce VEGF. Incubation of dendritic cells with VEGF and oncostatin M led to their transdifferentation into endothelial-like cells, expressing factor VIII-related antigen, VE-cadherin, and forming vascular-like tubes on Matrigel (Gottfried et al. 2007). Dendritic cells differentiated in the presence of adenosine express higher levels of VEGF, IL-6, IL-8, TGF-β, thus sustaining tumor angiogenesis (Novitiskiy et al. 2008). VEGF,

Table 4.2 Pro-angiogenic and anti-angiogenic molecules secreted by dendritic cells

Pro-angiogenic
Vascular endothelial growth factor (VEGF)
Interleukin-6 (IL-6)
Interleukin-8 (IL-8)
Transforming growth factor beta (TGF-β)
Tumor necrosis factor alpha (TNF-α)
CXCL-1, -2, -3, -5, -8
Osteopontin
Anti-angiogenic
IL-12
CXCL-9, 10
Interferon alpha (IFN-α)
Thrombospondin-1 (TSP-1)
Long pentraxin-3 (PTX-3)

IL-6, and TGF-β contribute to reduction of mature dendritic cells and accumulation of immature tolerant dendritic cells and polarization of dendritic cells toward Th2 or Treg induction, expression of activation of immunosuppressive mechanisms (Shurin et al. 2013).

TNF-α and CXCL-8 produced by immature dendritic cells in ovarian cancer ascites promote angiogenesis in vivo (Curiel et al. 2004) and OPN released from immature dendritic cells acts as a trigger for the production of the pro-angiogenic factor IL-1β from monocytes (Naldini et al. 2006). A population of $CD45^+$ $CD11^+$ $MHC\text{-}II^+$ dendritic cells infiltrating human ovarian carcinoma and expressing VE-cadherin, assemble into vascular tubes following implantation of tumor-derived $CD\text{-}45^+$-$VE\text{-}cadherin^+$ cells in immunodeficient mice (Cornejo-Garcia et al. 2004).

3 Lymphocytes and Platelets

T cell infiltration has been correlated with improved prognosis in colorectal cancer, breast cancer, renal cell carcinoma, melanoma, ovarian and gastrointestinal stroma tumors (GIST) (Gajewski et al. 2013). Th1 –cytotoxic cells may prevent tumors from growing locally (Fridman et al. 2012).

In ovarian cancer, CD4/CD25-positive Tregs secrete higher amounts of VEGF-A and promote EC proliferation in vitro and in vivo (Facciabene et al. 2011). During interaction with dendritic cells, activated CD4-positive T cells can acquire neuropilin-1, a co-receptor that bind VEGF from dendritic cells. The resulting neuropilin1-positive T cells bind dendritic cell-secreted VEGF-A and could behave as VEGF-carrying cells, promoting angiogenesis (Bourbie-Vaudanic et al. 2006).

Several studies have shown that tumor infiltrating NK (TINK) and tumor-associated NK (TANK) are compromised in their ability to lyse tumor cells; they are potentially pro-tumorigenic and can also acquire a pro-angiogenic phenotyoe (Bruno et al. 2014). Decidual NK cells are able to release angiogenic factors, including VEGF, PlGF, and IL-8, necessary for spiral artery formation during decidualization (Vacca et al. 2011). Tumor infiltrating NK cells in non small cell lung cancer (NSCLC) produce elevated levels of VEGF, PlGF, IL-8, and induce ex vivo ECs chemotaxis and tube formation (Bruno et al. 2013).

IL-12 receptors are expressed by NK and T cells (Trinchieri 1993). IL-12 suppresses angiogenesis through NK-mediated cytotoxicity of ECs (Yao et al. 1999) and IL-12-activated lymphocytes exert an anti-angogenic activity through the release of interferon gamma (IFN-γ) and down-modulation of VEGF (Cavallo et al. 2001).

Tumor cells express tissue factor on their plasma membranes which, in turn, provide a surface for pro-thrombinase activation; thus, intravascular clotting tumor cells provide the functions played by platelets in intravascular clotting (Dvorak et al. 1983). Moreover, tumor cells shed portions of their plasma membranes in the form of vesiscles (exosomes), which possess pro-coagulant activity (Dvorak et al. 1983).

Platelets contain a whole range of pro- and anti-angiogenic compounds, which are endocytosed and sequestered in different populations of alpha granules and accumulation of platelets in some tumors and release of angiogenic molecules could further stimulate tumor growth (Klement et al. 2009).

4 Mast Cells

Although some evidence suggest that mast cells can promote tumorigenesis and tumor progression, there are some clinical data as well as experimental tumor models in which mast cells seems to have functions that favor the host (Ribatti and Crivellato 2009). Mast cells attracted in the tumor microenvironment by stem cell factor (SCF) secreted by tumor cells, produce several angiogenic factors as well as MMPs, which promote tumor vascularization and invasiveness, respectively (Ribatti and Crivellato 2009). Mast cells are a major source of histamine, which modulate tumor growth through H1 and H2 receptors (Fitzsimons et al. 2007). H1 receptor antagonists significantly improved overall survival rates and suppressed tumor growth as well as the infiltration of mast cells and VEGF levels through the inhibition of HIF-1α expression in B16F10 melanoma-bearing mice (Jeong et al. 2013).

Mast cells exert immunosuppression releasing TNF-α and IL-10, which are essential in promoting the immune tolerance mediated by Treg cells, and stimulate immune tolerance and tumor promotion (Ullrich et al. 2007; Grimbaldesnton et al. 2007). Mast cells may promote inflammation, inhibition of tumor cell growth, and tumor cell apoptosis by releasing cytokines, such as interleukin IL-1, IL-4, IL-6, IL-8, monocyte chemotactic protein-3 and -4 (MCP-3 and MCP-4), TGF-β, and chymase. Chondrotin sulphate may inhibit tumor cells diffusion and tryptase causes both

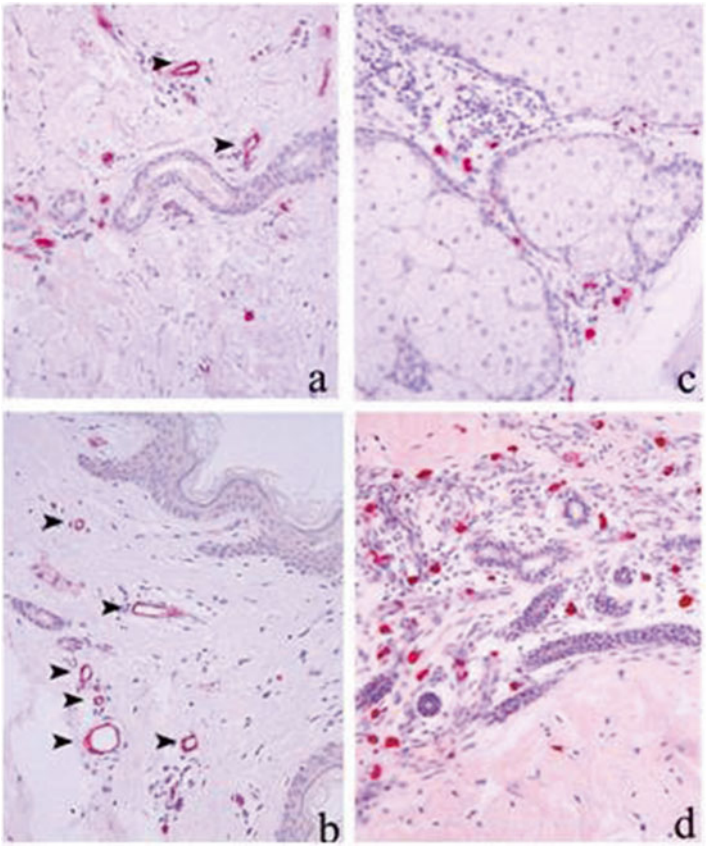

Fig. 4.5 Histological sections of human advanced primary melanomas stained with Factor VIII for microvessels (**a, b**) and with tryptase for mast cells (**c, d**) from patients with good (**a, c**) and poor prognosis (**b, d**) subgroup. Note the higher density of microvessels (*arrowheads*) and of mast cells (*red stained*) in poor prognosis patient (Reproduced from Ribatti et al. (2003a))

tumor cell disruption and inflammation through activation of protease-activated receptors (PAR-1 and -2) (Ribatti and Crivellato 2012a).

Within the developing tumor environment, mast cells do not act alone. They are recruited early in tumor development and play a critical role in both angiogenesis and tissue remodeling. As tumor growth progresses, mast cells recruit eosinophils and neutrophils and activate T and B cell immune responses (Kinet 2007). Data on mast cells and metastasis have been scant and poor. Yano et al. (1999) reported that mast cell infiltration around gastric cancer cells correlated with tumor angiogenesis and metastasis.

Increased mast cell number has been correlated with a poor prognosis in several human tumors, including melanoma (Fig. 4.5) (Ribatti et al. 2003a), oral squamous carcinoma (Wanachantatak 2003), and squamous cell carcinoma of the lip (Rojas et al. 2005).

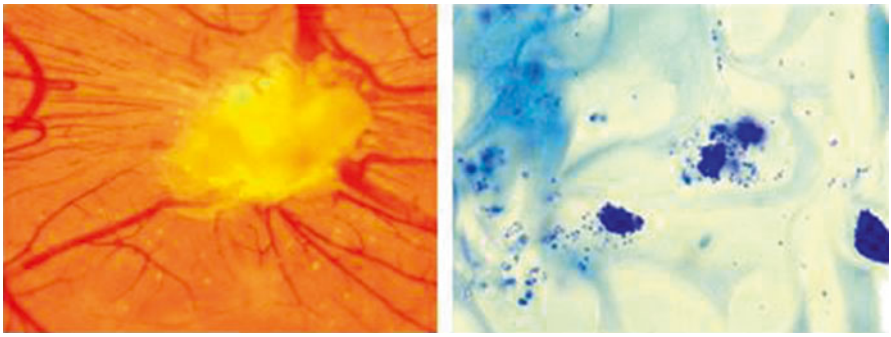

Fig. 4.6 A mast cell suspension has been delivered on the top of the chick embryo chorioallantoic membrane. Macroscopic observation shows the sponge surrounded by numerous allantoic vessels that develop radially towards the implant in a 'spoked-wheel' pattern. The histological analysis shows among the sponge trabeculae metachromatic mast cells and their secretory granules (Reproduced from Ribatti et al. (2001))

Mast cells produce several pro-angiogenic factors, including FGF-2, VEGF, IL-8, TNF-α, TGF-β, and nerve growth factor (NGF) (Qu et al. 1995, 1998a, b; Grützkau et al. 1998; Abdel-Majid and Marshall 2004; Boesiger et al. 1998; Kanbe et al. 2000; Moller et al. 1993; Walsh et al. 1991; Nilsson et al. 1997), migrate in vivo and in vitro in response to VEGF and placental growth factor-1 (PlGF-1) (Detmar et al. 1998; Gruber et al. 1995; Detoraki et al. 2009). Human lung mast cells express VEGF-A, VEGF-B, VEGF-C and VEGF-D, and supernatants of prostaglandin E2 (PGE2)- and 5′-N-methyl carboxamide adenosine (NECA)-activated lung mast cells induced angiogenic response in the chick embryo chorioallantoic membrane (CAM) assay that was inhibited by an anti-VEGF-A antibody (Detoraki et al. 2009). Granulated murine mast cells and their granules are able to stimulate an intense angiogenic reaction in the CAM assay, partly inhibited by anti-FGF-2 and -VEGF antibodies (Fig. 4.6) (Ribatti et al. 2001). Intraperitoneal injection of the degranulating compound 48/80 causes a vigorous angiogenic response in the rat mesentery window angiogenic assay and in mice (Norrby et al. 1986, 1989). Histamine and heparin stimulate proliferation of endothelial cells in vitro and are angiogenic in the CAM assay (Sörbo et al. 1994; Ribatti et al. 1987).

Mast cells store in their secretory granules pre-formed active serine proteases, including tryptase and chymase (Metcalfe et al. 1997). Tryptase stimulates the proliferation of ECs, promotes vascular tube formation in vitro, degrades connective tissue matrix, and activates matrix MMPs and plasminogen activator (PA), which in turn degrade the extracellular matrix with consequent release of VEGF or FGF-2 from their matrix-bound state (Blair et al. 1997). The expression of mast cell chymase and tryptase correlated with mast cell maturation and angiogenesis during tumor progression in chemically induced tumor growth in BALB/c mouse (de Souza et al. 2012). Mast cells contain MMPs, and TIMPs, (Tanaka et al. 2001; Koskivirta et al. 2006) which intervene in regulation of ECM degradation, allowing release of angiogenic factors.

Mast cell-deficient W/Wv mice exhibit a decreased rate of tumor angiogenesis (Starkey et al. 1988). Development of squamous cell carcinoma in a human papilloma virus (HPV) 16 infected transgenic mouse model of epithelial carcinogenesis provided experimental support for the early participation of mast cells in tumor growth and angiogenesis (Coussens et al. 1999, 2000). Mast cells infiltrated hyperplasia, dysplasias, and the invasive front of carcinomas, but not the core of tumors. Accumulation occurred proximal to developing capillaries and the stroma surrounding the advancing tumor mass (Coussens et al. 1999). Infiltration of mast cells and activation of MMP-9 coincided with the angiogenic switch in premalignant lesions through the release of pro-angiogenic molecules from the ECM. Remarkably, premalignant angiogenesis was abrogated in a mast cell-deficient HPV 16 transgenic mouse indicating that neoplastic progression in this model involved infiltration of mast cells in the skin (Coussens et al. 1999, 2000). By using the same in vivo transgenic mouse model, it has been demonstrated that genetic elimination of mature T and B lymphocytes, limits neoplastic progression (de Visser et al. 2005; Andreu et al. 2010). Moreover, in prostate tumors derived from both tumor transgenic adenocarcinoma of the mouse prostate (TRAMP) mice and human patients, mast cells promote well-differentiated adenocarcinoma growth (Pittoni et al. 2011).

An increased number of mast cells have been demonstrated in angiogenesis associated with vascular tumors, like haemangioma and haemangioblastoma (Glowacki and Mulliken 1982), as well as a number of haematological and solid tumors, including lymphomas (Ribatti et al. 1998; Fukushima et al. 2001), multiple myeloma (Ribatti et al. 1999) myelodysplastic syndrome (Ribatti et al. 2002), B-cell chronic lymphocytic leukemia (Molica et al. 2003; Ribatti et al. 2003b), breast cancer (Hartveit 1981; Bowrey et al. 2000), colon-rectal cancer (Lachter et al. 1995), uterine cervix cancer (Graham and Graham 1996; Benitez-Bribiesca et al. 2001; Ribatti et al. 2005a), melanoma (Dvorak et al. 1980; Reed et al. 1996; Ribatti et al. 2003a), and pulmonary adenocarcinoma (Ullah et al. 2012), in which mast cell accumulation correlate with increased neovascularization, mast cell VEGF and FGF-2 expression, tumor aggressiveness and poor prognosis (Tóth-Jakatics et al. 2000; Ribatti et al. 2003a). Indeed, a prognostic significance has been attributed to mast cells and microvascular density also in squamous cell cancer of the esophagus (Elpek et al. 2001). An association of VEGF and mast cells with angiogenesis has been demonstrated in laryngeal carcinoma and in small lung carcinoma (Sawatsubashi et al. 2000; Imada et al. 2000; Takanami et al. 2000; Tomita et al. 2000).

5 Macrophages

Cells belonging to the monocyte-macrophage lineage are a major component of the leucocyte infiltration in tumors (Balkwill and Mantovani 2002). Tumor-derived chemo attractants ensures macrophage recruitment, including colony stimulating factor-1 (CSF-1), the CC chemokines CCL-2, CCL-3, CCL-4, CCL-5 and CCL-8, and VEGF secreted by both tumor and stromal elements (Mantovani et al. 2002).

Activated macrophages are generally categorized in two types, M1 (classically activated) and M2 (alternatively activated) (Balkwill and Mantovani 2002). M1 macrophages are able to kill microorganisms as well as tumor cells and secrete high levels of pro-inflammatory cytokines and tumoricidal agents (TNF-α and IL-12), reactive nitrogen and oxygen intermediates (RNI, ROI) (Balkwill and Mantovani 2002). In the tumor microenvironment, TAMs are mainly constituted by M2 elements (Mantovani et al. 2002), which have poor attitude to destroy tumor cells but are better adapted to promoting angiogenesis, repairing and remodeling wounded or damaged tissues, and suppressing adaptive immunity (Sica et al. 2006). In regressing and non-progressing tumors, TAMs mainly resemble the M1 type and exhibit antitumor activity, while in malignant and advanced tumors, TAMs are biased toward the M2 phenotype that favors tumor malignancy (Qian and Pollard 2010). Unique cell surface markers that distinguish the two TAM phenotypes remain elusive and expression of M1/M-2 associated molecules is highly dependent on tumor type, tumor stage, intratumoral localization, hypoxia, and other microenvironmental signals (Mantovani et al. 2002). The phenotype of polarized M1-M2 macrophages has the potential to be reversed (Guiducci et al. 2005). Rolny et al. (2011) demonstrated that the host-produced histidine-rich glycoprotein (HRG) skewed TAM polarization away from the M2 to a tumor-inhibiting M1-like phenotype. In this context, HRG promoted antitumor immune responses and vessel normalization. Extensive TAM infiltration correlates with a poor prognosis for breast, prostate, cervix, and bladder cancer patients (Talmadge et al. 2007).

TAMs produce several pro-angiogenic cytokines, including VEGF, TNF-α, IL-8 and FGF-2 as well as extracellular matrix-degrading enzymes, including MMP-2, -7, -9, -12, and cycloxygenase-2 (COX-2) (Naldini and Carraro 2005; Klimp et al. 2001).

In an experimental model of subcutaneous melanoma, both angiogenesis and growth rate correlate with tumor infiltration by macrophages expressing angiotensin I receptor and VEGF (Egami et al. 2003). Lewis lung carcinoma cells expressing IL-1-β develop neovasculature with macrophage infiltration and enhance tumor growth in wild-type but not in MCP-1-deficient mice, suggesting that macrophage involvement might be a prerequisite for neovascularization and tumor progression (Nakao et al. 2005). In a murine model of mammary carcinoma, deficiency of macrophage colony-stimulating factor (M-CSF), a inductor of macrophage recruitment in tumor tissues, reduces progression to invasive carcinoma and metastasis (Lin et al. 2001). In polyoma middle-T (PyMT)-induced mouse mammary tumors, accumulation of macrophages in pre-malignant lesions precedes the angiogenic switch and the progression into invasive tumors (Lin et al. 2006, 2007).

Inhibition of macrophage homing into the tumor microenvironment delayed the angiogenic switch, whereas genetic restoration of macrophages rescued the vascular phenotype. Up-regulation of angiogenesis in TAMs is stimulated by hypoxia and acidosis (Bingle et al. 2002).

Activated macrophages synthesize and release inducible nitric oxide synthase (iNOS), which increases blood flow and promotes angiogenesis (Jenkins et al. 1995). Angiogenic factors secreted by macrophages stimulate migration of other accessory cells that potentiate angiogenesis, in particular mast cells (Gruber et al.

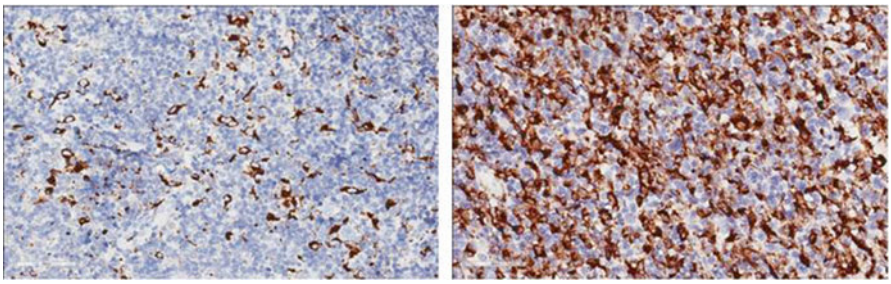

Fig. 4.7 A statistically difference was observed in the expression of CD68 in responders (*on the left*) and non-responders (*on the right*) groups of patients affected by diffuse large B-cell lymphoma (DLBCL) (Reproduced from Marinaccio et al. (2014))

1995). Osteopontin (OPN) deeply affects the pro-angiogenic potential of human monocytes (Denhardt et al. 2001), and may affect angiogenesis by acting directly on ECs and/or indirectly via mononuclear phagocyte engagement, enhancing the expression of TNF-α and IL-1-β in mononuclear cells (Leali et al. 2003; Naldini et al. 2006). Macrophages release IL-12, which cause tumor regression and reduce metastasis in animal models, through the promotion of anti-tumor immunity and also to the inhibition of angiogenesis (Colombo and Trinchieri 2002). The anti-angiogenic activity is mediated by IFN-γ production, which in turn induces the chemokine IFN-γ-inducible protein-10 (Angiolillo et al. 1995; Romagnani et al. 2001). Moreover, IL-12 inhibits VEGF production by breast cancer cells and regulates stromal cell interactions, leading to decreased MMP-9 and increased tissue inhibitor of TIMP-1 production (Dias et al. 1998).

Upregulation of angiogenesis in TAMs is stimulated by hypoxia and acidosis (Bingle et al. 2002). Mice deficient in HIF-2α in myeloid cells, displayed reduced TAM infiltration in both murine hepatocellular and colitis-associated colon carcinoma models (Imtiyaz et al. 2010). Mouse mammary tumors, exhibiting enriched M2-like TAMs in hypoxic tumor areas, demonstrated an increased angiogenic activity (Movahedi et al. 2010). The developing vasculature in tumors lacking myeloid-cell-derived VEGF-A was less tortuous, with increased PC coverage (indicating increased maturation), decreased vessel length, with evidence of vascular normalization and increased susceptibility to chemotherapeutic agents (Stockmann et al. 2008).

A significant relationship between the number of TAMs and the density of blood vessels has been established in human tumors, including breast carcinoma, melanoma, glioma, squamous cell carcinoma of the esophagus, bladder carcinoma, and prostate carcinoma (Leek et al. 1996; Makitie et al. 2001; Nishie et al. 1999; Koide et al. 2004; Hanada et al. 2000; Lissbrant et al. 2000). We have recently investigated CD68 expression and its relationship with microvascular density in chemo-resistant and chemosensitive patients affected by diffuse large B-cell lymphoma (DLBCL) and observed that CD68 expression as well as microvascular density were increased in chemo-resistant patients when compared with chemosensitive patients (Marinaccio et al. 2014) (Fig. 4.7).

Depletion of TAMs reduces to about 50 % tumor vascular density, leading to areas of necrosis by loss of blood supply within the tumor mass, and macrophages accumulate particularly in such necrotic and hypoxic areas in different tumors, like human endometrial, breast, prostate and ovarian carcinomas (Ohno et al. 2004; Leek et al. 1999).

6 Tie-2 Expressing Monocytes (TEMs)

The Tie-2 tyrosine kinase receptor has been shown to be expressed only by ECs and HSCs (Jones et al. 2001). Whereas only 1–2 % of total leukocytes are Tie-2$^+$, a substantial fraction (~20 %) of circulating monocytes express Tie-2 (Venneri et al. 2007; Murdoch et al. 2007). The majority of the circulating TEMs do not express EC/EPC markers, such as VEGFR-2, AC133, CD146 and CD34, whereas they express hematopoietic markers, such as CD45. Moreover, circulating human TEMs do not express CCR-2, the receptor for MCP-1, a chemokine that regulates the recruitment of monocytes to inflammed tissues and tumors. Thus, TEMs might be attracted to tumors in a CCR-2 independent manner, by signals produced by tumor cells, stromal cells or ECs. Indeed, Tie-2$^+$ CD14$^+$ cells isolated from human peripheral blood, and co-injected with human glioma cells s.c. in nude mice, promoted tumor vascularization whereas CD14$^+$ Tie-2$^-$ monocytes did not show this activity (Venneri et al. 2007). Human TEMs are preferentially recruited to tumors, where they constitute the prominent monocyte population distinct from TAMs.

TEMs have been observed in several mouse tumor models, including subcutaneous tumor grafts, spontaneous insulinomas developing in RIP-TAG2 transgenic mice, and human gliomas growing in the mouse brain, where they synthesize high levels of FGF-2 (De Palma et al. 2005). In these tumors, TEMs constituted a small population of the total tumor infiltrating CD11b$^+$ myeloid cells that could be distinguished from the majority of TAMs by their surface marker profile (Tie-2$^+$, Sca-1$^+$, CD11b$^+$) and their pro-angiogenic activity (De Palma et al. 2005). TEMs were found in human tumors including those of kidney, colon, pancreas and lung, as well as in soft tissue sarcomas, where were found both in perivascular and avascular viable (hypoxic) areas of tumors and were missing in non-neoplastic tissues adjacent to tumors (Venneri et al. 2007). Exposure to both hypoxia and Ang-2 markedly suppressed the release by TEMs of an anti-angiogenic cytokine, namely IL-12 (De Palma et al. 2003), and of TNF-α, which exterts a pro-apoptotic effect on both tumor cells and ECs (Balkwill 1992).

TEMs did not differentiate into ECs, suggesting that their pro-angiogenic activity could consist of a paracrine stimulation of angiogenesis. The selective elimination of TEMs by means of a suicide gene impaired angiogenesis in mouse tumors and induced substantial tumor regression and TEMs elimination did not affect the overall number of TAMs and granulocytes, indicating that TEMs represent a distinct monocyte subset with specific pro-angiogenic activity. Moreover, although recruited to tumors in lower numbers than TAMs, TEMs are a more potent source of pro-angiogenic signals.

De Palma et al. (2003) proposed that the percentage of incorporated EPCs into tumor vessels is very low. TEMs, while stimulating angiogenesis, do not actively incorporate into blood vessels and this subpopulation of Tie-2$^+$ cells, rather than bone marrow-derived EPCs, which are incorporated in new-forming blood vessels, promote tumor neovascularization through the release of pro-angiogenic factors.

TEMs may also play a role in angiogenesis in wound healing and in non-neoplastic diseases. In mice underwent partial hepatectomy 7–10 days earlier, TEMs were found in granulation tissue surrounding the regenerating hepatic lobules, also in proximity of newly-formed vessels, suggesting that they might also contribute to promote angiogenesis during liver regeneration (De Palma et al. 2003). TEMs were not observed in normal tissues suggesting that they may represent a specific subset of resident monocytes (Venneri et al. 2007).

The identification of specific molecules expressed by TEMs in tumors could facilitate the design of novel anticancer therapies that selectively target these cells. De Palma et al. (2008), by transplanting hematopoietic progenitors transduced with a Tie-2 promoter/enhancer-driven IFN-α-1 gene, turned TEMs into IFN-α cell vehicles that targeted the IFN response to orthotopic human gliomas and spontaneous mouse mammary carcinomas and obtained significant antitumor responses and near complete abrogation of metastasis.

7 Fibroblasts

Critical elements in the tumor microenvironment include TAFs, which provide an essential communication network via secretion of growth factors and chemokines, inducing an altered ECM thereby providing additional oncogenic signals that enhance cancer cell proliferation and spread. Fibroblasts are interconnected with tumor cells at all stages of cancer progression by promoting tumor growth, angiogenesis, and the metastatic process (Cirri and Chiarugi 2012).

TAFs are characterized by the expression of specific markers and secrete growth factors and angiogenic factors (Tables 4.3 and 4.4). TAFs modulate tumor growth by secreting: (i) growth factors able to increase tumor cell proliferation and exert an anti-apoptotic activity; (ii) chemotactic factors able to recruit other stromal cells, including leukocytes, monocytes/macrophages, and mast cells. Moreover, TAFs (type 1 polarized fibroblasts) induce immunosuppression by an increase in Th2 cells, Th17 cells and regulatory T cells, and are also involved in therapy resistance (Müerköster et al. 2004). Co-injection of TAFs, but not of normal fibroblasts, with tumor cells resulted in enhanced tumor formation (Hu et al. 2009; Augusten 2014).

TAFs (type 2 polarized fibroblasts) are predominantly assigned with a tumor promoting function under the influence of growth factors and chemokines. They stimulate cancer cell survival, growth, and invasion, contribute to angiogenesis, and impact the activations state of various immune cells (Augusten 2014).

TAFs were increased in patients with active MM compared to those in remission and those with MGUS (Frassanito et al. 2014). The cells displayed an activated

Table 4.3 Markers expressed by tumor associated fibroblasts

Vimentin
Fibroblast specific protein 1 (FSP1)
Fibroblast activating protein (FAP)
Alpha smooth actin (αSma)
Thy1 (CD90)
Thrombospondin-1 (TSP-1)
Platelet derived growth factor receptor α/β (PDGFR α/β)
Tenascin-c (TN-C)
Matrix metalloproteinase-3 (MMP-3)
Podoplanin
Neuron glial antigen 2 (NG2)
Periostin
Palladin

Table 4.4 Growth factors and angiogenic factors secreted by tumor associated fibroblasts

Interleukin-6 (IL-6)
Insulin-like growth factor (IGF)
Hepatocyte growth factor (HGF)
Fibroblast growth factor-2 (FGF-2)
CXCL-8 (IL-8)
CXCL-12

phenotype, and produced high levels of TGF-β, IL-6, SDF-1α, and insulin-like growth factor (IGF-1). Moreover, they showed a heterogeneous phenotype which entailed their origin from resident fibroblasts, and from ECs and HSCs via endothelial-mesenchymal transition, and from MSCs via mesenchymal transition, all induced by both TAMs and MM plasma cells. Active MM fibroblasts favored chemotaxis, adhesion, proliferation and apoptosis-resistance of MM cells through cytokines and cell-to-cell contact that was inhibited by blocking CXCR4, several integrins, and fibronectin. Finally, studies in syngeneic 5T33MM and xenografted mouse models showed that MM cells induced the TAFs recruitment and expansion which, in turn, favored tumor initiation and progression as well as angiogenesis (Frassanito et al. 2014).

8 Osteoblasts and Osteoclasts

Osteoblasts derived from MSCs following timely programmed steps requiring the expression of specific genes, including bone morphogenetic proteins (BMPs) and Wnt pathways (Grigoriadis et al. 1988). Osteoblasts express the pro-angiogenic factor OPN and Ang-1 at the bone surface that are required for osteoblast-mediated HSCs maintenance (Arai et al. 2004). Ang-1 binds Tie2 expressed by BM-HSCs, inducing the activation of β1 integrin and N-cadherin and contributing to the

activation of HSCs (Arai et al. 2004). Moreover, Ang-1 is expressed by MM plasma cells and is associated with up-regulated expression of its specific receptor Tie-2 on the bone marrow ECs and enhanced angiogenesis (Giuliani et al. 2003). OPN is involved in the interplay between HSCs and osteoblast niche and inhibits HSCs proliferation (Nilsson et al. 2005).

Osteoblasts/HSCs interactions lead to the expression of receptor activator of RANKL and Notch activation (Yin and Li 2006). A small subset of spindle-shaped osteoblasts lining cells expressing N-cadherin, are the niche for HSCs in the BM (Zhang et al. 2003), and an increase in their number is correlated with an increase in HSC number (Calvi et al. 2003).

VEGF acts as a potent chemoattractant for osteoclasts (Mayr-Wohlfart et al. 2002), that regulate VEGF expression by ECs through the release of TGF-β from the bone matrix and its activation during bone resorption (Shao et al. 2009). Moreover, osteoclasts release heparinase that degrades heparin sulphate proteoglycans releasing VEGF and FGF-2, which contributes to aultocrine osteoclast activation and paracrine stimulation of osteoblast activity (Collin-Osdoby et al. 2002; Padera et al. 1999; Saijo et al. 2003). Osteoclasts release also BMP-7, which promotes EC survival and increases VEGF production (Garimella et al. 2008), and amphiREGulin, epiREGulin, and NeuREGulin, that induce EC migration and tube formation (Mehta and Besner 2007). Secretion of MMP-9 by osteoclasts enhances angiogenesis through the release of VEGF from the ECM (Cackowski et al. 2009).

Inhibition of osteoclasts reduces angiogenesis and tumor burden in MM (Croucher et al. 2003). Osteoclasts secrete OPN, which cooperates with VEGF of MM plasma cells to enhance angiogenesis and induce osteoclastogenic activity by ECs (Tanaka et al. 2007). Moreover, in combination with IL-6, OPN enhances MM plasma cell growth (Abe et al. 2004). OPN knockout mice display minimal bone resorption compared with wild type and decreased osteoclast association at the bone surface (Asou et al. 2001).

9 Cancer Stem Cells (CSCs)

CSC may be defined as "a small substet of cancer cells within a cancer that constitute a reservoir of a self-sustaining cells with the exclusive ability to self-renew and to cause the hetereogenous lineages of cancer cells that comprise the tumor" (Clarke et al. 2006).

The property of self-renewal is a hallmark of CSC. Mechanisms involved in self-renewal are dysregulated and lead to CSC overpopulation. Tumor cells in any cancer may be organized as a hierarchy than can be roughly classified as long term CSC (LT-CSC), short-term CSC (ST-CSC), early and late tumor progenitors, and differentiating and differentiated tumor cells. The LT-CSC should possess indefinite self-renewal and ST-CSC some self-renewal properties. So, CSC like normal SC give rise to hierarchical organization of cell populations that, in turn, produce rapidly proliferating cells, resulting in the generation of fully differentiated cells (Reya

Table 4.5 Angiogenic markers expressed by cancer stem cells

Tumor type	CSC marker	Angiogenic marker
Malignant glioma	CD133	VEGF
Glioblastoma	Nestin	VEGF
Melanoma	CD133, ABCG2[a]	VEGF, VEGFR-2, Ang-1, Ang-2, Tie2
Oligodendroglioma	CD133, Nestin	CD34
Pancreatit cancer	CD133	VEGF-c
Hepatocellular carcinoma	CD133, ABCG2, Nestin, CD44	VEGF, PDGF

[a]ABCG2, ATP-binding cassette subfamily G member 2

et al. 2001). At the top of the hierarchy there is a CSC that expresses surface markers of primitive cells and possesses differentiation potential and limitless proliferative potential.

CD133/AC133 was discovered as a marker of normal HSCs and later was found to mark SCs from a wide variety of tissues (Shmelkov et al. 2005). CD133 was used to purify putative CSC in different tumors (Boman and Wicha 2008). CD44 and CD24 have been proposed as markers for CSC several tumors; however, while some literature data indicated the existence of CD44+/CD24- cells as CSCs in breast, prostate, colon, pancreatic, and hepatic carcinoma, and melanoma (Wicha et al. 2006; Dalerba et al. 2007), other works demonstrated that the lack of CD24 expression is not an essential feature of CSC. In fact, a subpopulation of CD24+ cells possesses SC characteristics in colon and pancreatic carcinoma (Ke et al. 2012; Zhang et al. 2012). CD20 has been proposed as a marker in melanoma and MM; CD166 in colon carcinoma; aldehyde dehydrogenate 1A1 (ALDH1) in breast, prostate, and pancreatic carcinomas, leukemia and multiple myeloma; epidermal surface antigen (ESA) in breast, colon, and pancreatic carcinomas (Boman and Wicha 2008). ABCG2, a member of the ABC transporters, which pump a variety of endogenous and exogenous compounds out of cells, is recognized as a universal marker of SC and plays an important role in promoting SC proliferation and the maintenance of the SC phenotype. Moreover, ABCG2 is a marker of CSC (Ding et al. 2010). In brain tumors, CD133+ but non CD133- cells, grow in aerosphere conditions, express a number of neuronal SC markers, are highly tumorigenic and resistant to radiation (Singh et al. 2003; Lee et al. 2006). CSCs express also angiogenic markers (Table 4.5).

CSC markers could be used for prediction treatment responses by means of the identification of the presence of specific CSC subtypes sensitive to the therapeutic agents and have a prognostic value by allowing assessment of the size of the CSC population within any give tumor. However, no defined localization patterns have been established when the in situ distribution of CSC markers is evaluated. In addition, the markers used to sort putative cancer-initiating cells, including CD24, CD44, and CD133, are also expressed on normal cells (Clarke et al. 2006).

The demonstration that tumor growth depends on a subpopulation of proliferating CSC was first described in transmittable leukemias of mice. Furth and Kahn

(1937) were able to transplant leukemia from one mouse to another using a single undifferentiated cell and Makino and Kano (1955) obtained clones of tumor cells from single cells. Researchers started to search for stem-like cells in leukemias by testing the ability of various purified populations to form leukemia in non-obese diabetic/severe combined immunodeficiency (NOD-SCID) mice. Injection of leukemic cells with primitive hematopoietic progenitors resulted in leukemias that could be serially transplanted into secondary recipients, whereas injection of more differentiated leukemic cells did not (Bonnet and Dick 1997). Kelly et al. (2007) used two transgenic mouse models in which the Eμ enchancer was used to express either the *c-myc* or *N-ras* oncogenes to induce B or T cell lymphomas. They concluded that "tumor growth need not be driven by rare cancer stem cells" based upon >10 % of transplanted cells give rise to tumors in syngeneic mice. Guo et al. (2008) using a mouse model of human leukemia in which depletion of the *Pten* tumor suppressor gene in HSC resulted in a myeloproliferative disorder followed by acute T lymphoblastic leukemia demonstrated that population of leukemia SCs was responsible for leukemia development.

CSCs have been identified in several human solid tumors, including melanoma, brain, colorectal, hepatic, head and neck, breast and prostate cancer (Reya et al. 2001; Singh et al. 2003; Lee et al. 2006), and are able to initiate tumor formation and metastasis and there is a growing body of evidence suggesting that metastases develop when distant organs are seeded with CSCs that arise from a primary tumor (Balic et al. 2006). Hermann et al. (2007) identified a subset of $CD133^+/CXCR4^+$ pancreatic CSCs located at the invasive front of tumors, which seem to be more responsible for metastasis.

The frequency of functionally defined CSCs vary among different patients and in some cases are relatively rare, whereas in others can constitute a substantial proportion of the tumor mass (Quintana et al. 2008). Glioblastoma multiforme contains neural precursors endowed with all the critical features from neural SCs (Galli et al. 2004). When neurospheres derived from highly vascularized glioblastomas were transplanted into mice, the initial tumors demonstrated low-grade glioma phenotype without any sign of angiogenesis. Upon serial transplantation, the tumor cells developed a highly malignant phenotype with extensive angiogenesis and necrosis in tumor masses (Sakariassen et al. 2007).

CSCs that express markers of vascular endothelium and form tumor blood vessels have been described. Brain tumor ECs interact with self-renewing brain tumor cells and secrete factors that maintaining them in a 'stem cell-like' state. An increased number of ECs promotes the expansion of self-renewing SC and accelerates tumor growth, while their depletion has the opposite effect (Frank et al. 2010; Stupp and Hegi 2007). Calabrese et al. (2007) established a co-culture system to investigate the interaction between vascular ECs and CSCs and demonstrated that ECs interact with $Nestin^+/CD133^+$ CSCs and provide factors that support their self-renewal mechanisms, demonstrating that the vasculature is essential in supporting and preserving the survival, stem-like properties, and functions of CSCs. Moreover, Calabrese et al. (2007) showed that bevacizumab can reduce the stem-like cell fraction in brain tumors.

Ricci-Vitiani et al. (2010) demonstrated that in vitro culture of glioblastoma stem-like cells (GSC) in conditions favorable to an EC differentiation generated a progeny with phenotypic and functional features of ECs. Moreover, orthotopic or subcutaneous injection of GSCs in immunocompromised mice generated large anaplastic tumor xenografts, showing a vessel wall formed by human ECs derived from GSCs whereas tumor derived ECs formed large anaplastic tumors in secondary recipients. Finally, selective targeting of ECs generated by GSC in mouse xenografts resulted in tumor regression, suggesting a functional relevance of GSC-derived ECs. Accordingly to these data, Wang et al. (2010) demonstrated that GSCs can give rise to ECs or tumor cells, through an intermediate $CD133^+/CD144^+$ progenitor cell. CSCs produce much higher levels of VEGF in both normal and hypoxic conditions than non-CSC population, leading to a strong angiogenic response (Bao et al. 2006).

CSCs may represent a plastic entity whose phenotype and function are continuously modulated by the tumor microenvironment, epigenetic regulation, and different experimental systems. Accordingly, Quintana et al. (2008) showed that modified xenotransplantation assay conditions, including the use of more highly immunocompromised NOD-SCID IL-2 receptor gamma chain null mice, can increase the detection of tumorigenic melanoma cells by several orders of magnitude. CXCR4 expression by a subset of CSCs might be responsible for the signals that CSCs receive from the local microenvironment, determining which cancer cell possesses the capacity to self-renewal (Hermann et al. 2007). Moreover, CSC angiogenic potential may differ depending on the stage of tumor progression. Accordingly, more primitive cells may play a more significant role at an early stage when the tumor is smaller, whereas more mature cells may be involved as the tumor increases in size.

CSCs may reside in a vascular niche in close proximity to blood vessels (Borovski et al. 2011) named as 'cancer stem cell niche' (Calabrese et al. 2007). Relationship between CSCs and their niche may be bi-directional: CSCs generate angiogenic factors to stimulate angiogenesis and the tumor vasculature supports maintaining CSC self-renewal and maintenance. Inhibition of vessel growth may destroy the niche and reduce the number of CSCs (Yang and Wechsler-Reya 2007).

Vascular niche may be considered as a specialized microenvironment that, through paracrine signaling interactions, control CSC proliferation and fate determination. The activation by angiogenic factors and inflammatory cytokines switch the "vascular niche" to promote tumor growth (Butler et al. 2010). Bao et al. (2006) observed that GSCs $CD133^-$ are located near to blood vessel, while SCLGC $CD133^+$ cells generated high vascularized tumors expressing 10–20 fold more VEGF than the $CD133^-$ fraction. Moreover, anti-VEGF antibody bevacizumab depleted SCCGC-induced vascular EC migration and tube formation and inhibited the tumor growth of SCLGC-derived xenografts. Folkins et al. (2009) demonstrated that tumor with larger SCLGC population recruited a higher amount of EPCs suggesting that SCLGC promote angiogenesis and EPC mobilization through release of VEGF and SDF-1. Calabrese et al. (2007) demonstrated that oligodendroglioma and GSCs, which expressed the major intermediate filament protein nestin, are located in close proximity to CD34+ capillaries and are positively correlated to microvascular

density. These findings strongly support the hypothesis that CSCs are located within a "vascular niche".

CSC are more resistant to chemotherapeutic agents and radiation therapy than more mature cell types from the same tissue because of increased expression of antiapoptotic proteins (Frank et al. 2010; Calabrese et al. 2007). Even if therapy kills all proliferating cells, SCs repopulate tumors. Radioresistance, chemotherapy resistance and angiogenesis mediated by CSCs could partially explain tumor recurrence in more aggressive human tumors. Inhibition of angiogenesis alone may not be sufficient to eradicate CSCs, even if anti-angiogenic therapy may sensitize CSCs to cytotoxic effects of chemotherapy.

Treatment of mice transplanted with human acute myeloid leukemia (AML) cells with anti-CD44 or anti-CXCR4 antibodies markedly reduced leukemic repopulation by CSCs (Jin et al. 2006; Tavor et al. 2004). When these monoclonal antibodies were injected into NOD/SCID mice repopulated with AML, the leukemic SCs were markedly depleted and in some cases, the mice were cured (Jin et al. 2006; Tavor et al. 2004). BMP-4 inhibits the tumorigenic potential of glioblastoma CSC through the BMP-4/BMPR/SMAD signaling pathway indicating that BMP play a critical role in CSC tumorigenesis and angiogenesis (Piccirillo et al. 2006). Notch inhibitor DAPT gamma secretase inhibitor leads to a reduction of self-renewal ability of CSCs and the number of $CD133^+$ tumor cells and of vascular markers, including CD105, CD31, and von Willebrand factor (Hovinga et al. 2010).

It is possible to treat cancer by inducing differentiation of CSCs, i.e. differentiation therapy (Spira and Carducci 2003). In general, SC differentiation results in two types of changes: the expression of specialized, differentiation-specific gene products and a partial or complete restriction of the cell's capacity for further proliferation. The most thoroughly examined and clinically tested as differentiating agents are retinoic acid (RA, vitamin A), in particular all-trans-retinoic acid (ATRA). Currently, about 90 % of newly diagnosed patients with acute promyelocytic leukemia (APL) achieve complete remission and over 70 % are cured by ATRA therapy (Camacho 2003) with or without concomitant chemotherapy with methotrexate and cytarabile (Ohno et al. 2003). Moreover, retinoids not only induce differentiation in leukemia, but they also regulate anti-apoptotic genes (Andreeff et al. 1999).

10 Circulating Endothelial Cells and Endothelial Precursor Cells

Normal adults have a low number of circulating ECs per ml of peripheral blood (Solovey et al. 1997). Most of these cells are quiescent, and at least half are microvascular as defined by CD36 positivity (Solovey et al. 1997). Circulating ECs are detectable in diseases marked by vascular injury, including sickle cell anemia, acute myocardial infarction, thrombotic thrombocytopenic purpura, and active cytomegalovirus infection (Hladovec et al. 1978; Grefte et al. 1993; Lefevre et al.

1993; Solovey et al. 1997). Immature EPCs from primitive HSCs share common surface, i.e. AC-133, CD34 or VEGFR-2. There is no exclusive EPC marker. Both EPCs and mature ECs express similar endothelial-specific markers, including VEGFR-2, Tie-1, Tie-2 and VE-cadherin (Sato et al. 1993; Schnurch and Risau 1993; Vittet et al. 1996; Eichmann et al. 1997). Moreover, HCs subsets express markers similar to those of EC, including CD34, PECAM, Tie-1, Tie-2, Eph and VEGR-1, transcription factors such as SCL/tal-1 and AML1, von Willebrand factor (vWF) (Suda et al. 2000; Lyden et al. 2001).

Asahara et al. (1997) showed that $CD34^+$, VEGFR-2^+ circulating EPCs form colonies that take up acetylated LDL. When $CD34^+$, $VEGFR2^+$, $CD34^-$ or VEGFR-2^- cells were injected into mice, rats, and rabbits undergoing neovascularization due to hind limb ischemia, $CD34^+$ and VEGFR-2^+ cells, but rarely $CD34^-$ or VEGFR-2^- cells, incorporated into the vasculature in a manner consistent with their being EC (Asahara et al. 1997). AC 133 is also expressed on EPC subsets, but not on mature ECs (Yin et al. 1997). Its expression is rapidly downregulated as HSCs and EPCs differentiate (Yin et al. 1997; Miraglia et al. 1997). Peichev et al. (2000) demonstrated that a small subset of $CD34^+$ cells from different hematopoietic sources express both AC 133 and VEGFR-2. Incubation of this subset with VEGF, FGF-2 and collagen resulted in their proliferation and differentiation into AC 133^- VEGFR-2^+ mature ECs. Maturation and in vitro differentiation of these cells abolish AC 133 expression, suggesting that EPCs with angioblast potential may be marked selectively with AC 133.

Gehling et al. (2000) demonstrated that $AC133^+$ cells from G-CSF-mobilized peripheral blood differentiate into ECs when cultured in the presence of VEGF and stem cell growth factor. Phenotypic analysis revealed that most of these cells display endothelial features, including the expression of VEGFR-2, Tie-2 and vWF. AC 133 is the best selective marker for identifying EPCs and circulating $CD34^+$ cells VEGFR-2^+ and $AC133^+$ constitute a phenotypically and functionally distinct population of circulating ECs that may play a role in postnatal vasculogenesis. AML1 is expressed in ECs in sites where early HSCs emerge, such as the yolk sac (North et al. 1999), and AML1-deficient embryos, which lack definitive hematopoiesis, display defective angiogenesis in the head and pericardium (Takakura et al. 1998).

Most circulating EPCs reside in the bone marrow in close association with HSCs and the stroma. When EPC are mobilized from adult BM, they first migrate from a quiescent niche within the BM into a permissive, proliferative microenvironment, the so-called vascular zone of marrow. MMP-9 as well as the survival/mitogenic activity of the stem cell cytokine SkitL are critical during this process. Release of EPCs from BM into circulation can be induced by GM-CSF or VEGF and is dependent on the activity of endothelial nitric oxide synthase (eNOS) expressed by stromal cells in BM (Aicher et al. 2003). Statins, erythropoietin and estrogen also positively influence EPC number (Dimmeler et al. 2001; Vasa et al. 2001; Heeschen et al. 2003; Strehlow et al. 2003).

EPCs in the peripheral blood may derive from the BM and be not yet incorporated into the vessel wall. The following lines of evidence suggest that they constitute the preponderance of circulating BM-derived endothelial lineage cells. Asahara

et al. (1999), employed a BM transplant model in mice sublethally irradiated to demonstrate the incorporation of BM EPCs into foci of neovascularization. Four weeks after transplants, when the BM had been reconstituted, a variety of surgical experiments to provoke neovascularization were performed: (i) Cutaneous wounds examined 4 and 7 days after skin removal by punch biopsy disclosed a high frequency of incorporated EPCs. (ii) One week after the onset of hind limb ischemia, EPCs were incorporated in capillaries among skeletal myocytes. (iii) After permanent ligation of a coronary artery, myocardial infarction sites demonstrated incorporation of EPCs in foci of neovascularization at the border of the infarct.

Shi et al. (1998) implanted an impermeable Dacron graft made in the canine thoracic aorta and demonstrated scattered islands of ECs without any evidence of transmural angiogenesis. Later, they used a transplantation model in which marrow cells from donors and recipients were distinguishable to determine whether ECs lining a vascular prosthesis are derived from the BM (Shi et al. 1998). After 12 weeks, the graft was retrieved, and cells with endothelial morphology were identified; only donor alleles were detected in DNA from positively stained cells on the graft. These results suggested that a subset of $CD34^+$ cells located in the BM mobilize to the peripheral circulation and colonize the endothelial flow surface of vascular prostheses.

Lin et al. (2000) found a distinction between vessel wall and BM-derived ECs in blood samples from subjects who had received gender-mismatched bone marrow transplants 5–20 months earlier. They showed that 95 % of circulating ECs had recipient genotype and 5 % had donor genotype. After 9 days of culture, ECs derived predominantly from the recipient vessel wall, expanded only sixfold, compared with 98-fold after 27 days by ECs, mostly originated from donor BM cells. These data suggest that most circulating ECs in fresh blood originate from vessel walls and have limited growth capability, and that outgrowth of ECs is mostly derived from transplantable marrow-derived cells. In animal models, EPCs home in on sites of active neovascularization and mobilization of BM-derived EPCs, and differentiate into ECs in response to tissue ischemia (Takakura et al. 1998). This finding is consistent with postnatal vasculogenesis.

Identification of the chemokines that induce mobilization of BM EPCs to the peripheral circulation may provide a novel mechanism for their recruitment to sites of vascular trauma to accelerate vascular healing (Rafii 2000). Moreover, expansion and mobilization of EPCs may augment the resident population of ECs competent to respond to exogenous angiogenic cytokines (Isner and Asahara 1999). Kalka et al. (2000) have demonstrated that transplantation of EPCs to athymic mice with hind limb ischemia markedly improves blood flow recovery and capillary density in the ischemic limb and significantly reduces the rate of limb loss.

The recruitment of EPCs to tumor angiogenesis is a multistep process, including: (a) active arrest and homing of the circulating cells within the angiogenic microvasculature; (b) transendothelial extravasation into the interstitial space; (c) extravascular formation of cellular clusters; (d) creation of vascular sprouts and cellular networks; (e) incorporation into a functional microvasculature.

High VEGF levels favour and enhance differentiation of HSCs toward the EPC lineage (Takahashi et al. 1999a, b; Gillies et al. 2008). High levels of VEGF produced by tumors may result in the mobilization of BM-derived SCs in the peripheral circulation and enhance their recruitment into the tumor vasculature (Asahara et al. 1999; Hattori et al. 2000). Moreover, Hattori et al. (2000) showed that combined elevation of VEGF and Ang-1 result in remodeling of the bone architecture, with depletion of the sinusoidal spaces of hematopoietic cells and a parallel increase in BM vascularization and splenomegaly. Hypoxia can also mobilize EPCs from the BM in the same way as hematopoietic cytokines, such as GM-CSF (Takahashi et al. 1999a, b). Malignant tumor growth results in neoplastic tissue hypoxia, and may mobilize BM-derived ECs in a paracrine fashion and thus contribute to the sprouting of new tumor vessels. Lyden et al. (2001) demonstrated that transplantation and engrafment of β-galactosidase-positive wild-type BM or VEGF-mobilized SCs into lethally irradiated Id-mutant mice is sufficient to reconstitute tumor angiogenesis. In contrast to wild type mice, Id-mutants fail to support the growth of tumors because of impaired angiogenesis. Tumor analysis demonstrates the uptake of BM-derived VEGFR-2$^+$ EPCs into vessels surrounded by VEGFR-1$^+$ myeloid cells. Defective angiogenesis in Id-mutant mice is associated with impaired VEGF-induced mobilization and proliferation of the BM precursor cells. Inhibition of both VEGFR-1 and VEGFR-2 signaling is needed to block tumor angiogenesis and induce necrosis.

Vajkoczy et al. (2003) propose the term "angiomorphosis" as the process of enhancing tissue vascularization through active recruitment of EPCs from the circulation by ECs and the action of EPCs as organizers of the angiogenic process.

11 Multipotent Adult Progenitor Cells (MAPCs) and Myeloid-Derived Suppressor Cells (MDSCs)

Reyes et al. (2002) have identified a single cell in human and rodent postnatal marrow that they term the MAPCs. MAPCs were selected by depleting adult BM of HC expressing CD45 and glycophorin-A, followed by long-term culture on fibronectin with epidermal growth factor (EGF) and PDGF under low serum conditions. A cell population expressing AC133 and low levels of VEGFR-1 as well as VEGFR-2 and the embryonic stem cell marker Oct-4 emerged. Its culture with VEGF induced differentiation into CD34$^+$, VE-cadherin$^+$, VEGFR-2$^+$ cells, a phenoptype consistent with angioblasts. Subsequently these cells express vWF and markers of mature endothelium, such as CD31, CD36 and CD62-P. MAPCs form vascular tubes when plated on Matrigel and upregulate angiogenic receptors and VEGF in response to hypoxia.

MDSCs have been characterized by cell surface co-expression of CD11b and Gr1 (Gabrilovich and Nagarajas 2009). In cancer, MDSCs undergo abnormal expansion and accumulate in different sites, including BM, spleen, liver, and tumor site sustaining tumor-derived inflammation and inducing tumor progression, neo-

vascularization and immune escape. MDSCs are mobilized during tumorigenesis and infiltrate developing tumors where they promote vascularization (Talmadge and Gabrilovich 2013) and disrupt major mechanisms of immunosurveillance (Gabrilovich et al. 2012). An elevated number of peripheral MDSCs correlate with advance disease and therapeutic inefficacy (Diaz-Montero et al. 2009).

MDSCs are one of the major components of the immunosuppressive network responsible for the impairment of the NK cell and T cell-dependent anti-cancer immunity and promote angiogenesis, cancer cell invasion, and metastasis (Monu and Frey 2012; Gabrilovich and Nagarajas 2009). The immunosuppressive effects of MDSCs are exerted through activation and expansion of the Treg population, interference with lymphocyte trafficking, creation of oxidative stress and depletion of nutrients (Gabrilovich et al. 2012). GM-CSF, VEGF, IL-3 promote blockade of myeloid maturation with an expansion in the MDSC population. Combined treatment with anti-Gr1 and anti-VEGF results in more pronounced reduction in the vascular surface area compared with anti-VEGF treatment alone (Shojaei et al. 2007).

MDSCs and Treg cells are mobilized into the circulation in response to activated cytokines that are induced by tumorigenesis, including TGF-β and CXCL5-CXCR2. MDSCs and Treg cells infiltrate the growing tumor to disrupt immune surveillance through multiple mechanisms, including disruption of antigen factors, secrete ECM proteins and BM components, regulate differentiation, modulate immune responses and contribute to deregulated homeostasis.

The identification of molecular pathways involved in MDSC function will lead to the development of new agents that disrupt the tumor-host interactions, thus improving the efficacy of immunotherapy.

Chapter 5
Therapeutic Strategies, the Concept of "Normalization" and the Role of VEGF Inhibition

Abstract There are a number of anti-angiogenic agents in clinical development showing varying degree of antitumor activity. The most promising include bevacizumab, the humanized anti-monoclonal antibody anti-VEGF approved for use in combination with cytotoxic agents, as well as small molecule receptor tyrosine kinase inhibitors (RTKIs). Targeting inflammatory processes as anti-angiogenic strategy in cancer can be beneficial. The normalization of tumor blood vessels by anti-angiogenic drugs is probably transient, and dependent on the dose and duration of the treatment.

Keywords Biphosphonate compound • Botrezomib • Cediranib • COX-2 inhibitor • Lenalidomide • Liposomes • Lymphangiogenesis • Non-steroidal anti-inflammatory drugs • Placental growth factor • Rapamycin • Thalidomide • Therapeutic strategies • Vascular normalization • Vinblastine • Zoledronic acid

With more than a million people treated with VEGF pathway inhibitors worldwide, anti-angiogenic treatment represents the most validated example that disruption of the tumor environment is an effective strategy for cancer treatment. Unfortunately, the stroking benefits of anti-angiogenic therapy observed in treating mouse tumors have not been translated to the clinic (Table 5.1). All of these drugs have only modest effects on human cancers. Despite significant improvement of clinical outcome in combination with chemotherapy, survival benefits of anti-angiogenic drugs in combination settings remain modest in most cancer types (Kerbel 2008).

The patient's benefit for the treatment is not satisfactory because of transient and modest performance of the anti-angiogenic agents in the clinic, off target toxicities (Table 5.2) and intrinsic refractoriness. These agents used as monotherapy or in combination with chemotherapy have only provided survival benefits on the order of weeks to months in some tumor types and have not been efficacious in all the others.

Moreover, anti-angiogenic therapy has been reported to cause an increased metastatic phenotype, through increased tumor hypoxia and hypoxia-induced EMT (Paez-Ribes et al. 2009; Ebos et al. 2009).

Anti-angiogenic drugs are delivered to cancer patients by systemic administration which may affect healthy vasculature distributed in multiple organs (Cao 2010).

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Table 5.1 Differences between human cancer and mouse tumor models

Features	Human	Mouse
Assessment	Survival	Tumor size
Tumor site	Intrinsic	Artificial
Tumor growth rate	Slow	Fast
Age	Old	Young
Genetic background	Heterogeneous	Homogeneous
Treatment	Begins at the advantage stage	Begins at the early stage

Table 5.2 Toxicities due to anti-angiogenic therapy

Cardiovascular
Hypertension
Thromboembolic disease
Left ventricular dysfunction
Myocardial ischemia
Non-cardiovascular
Proteinuria
Bleeding
Delayed wound healing
Gastrointestinal perforation
Fatigue
Thyroid dysfunction
Stomatitis
Myelosuppression
Cutaneous effects
Disphonia

Optimization of anti-angiogenic therapy involves the development of drug delivery systems that enable kinetic personalized therapy (Cao 2010).

Since March 2008, bevacizumab has been approved for treating patients with late-stage colon cancer, non-small cell lung cancer and breast cancer, all in combination with chemotherapy. The Food and Drug Administration (FDA) removed breast cancer because of the absence of clear benefit on the overall survival.

Two kinase inhibitors (Table 5.3), sorafenib and sunitinib, targenting VEGFRs tyrosine kinases, have both approved for treating renal carcinoma. In addition, sunitinib has been approved for treating gastrointestinal tumors, and sorafenib for hepatocellular carcinomas.

Targeting inflammatory processes as anti-angiogenic strategy in cancer can be beneficial. COX-2 expressing tumor cells are associated with the production of angiogenic growth factors and the synthesis and activation of MMPs, favoring tumor invasion and angiogenesis (Tsujii et al. 1997; Takahashi et al. 1999b). COX-2 itself has a pro-angiogenic activity (Macarthur et al. 2004), upstreams of VEGF production in stromal cells (Boccaccio et al. 2005), and catalyzed prostaglandin (PGE)-2 expression playing a critical role in the HIF-1α-mediated regulation of

Table 5.3 Registered kinase inhibitors

Imatinib (Glivec, Gleevec)
Gefitinib (Iressa)
Erlotinib (Tarceva)
Lapatinib (Tykerb)
Dasatinib (Sprycel)
Nilotinib (Tasigna)
Sunitinib (Sutent)
Sorafenib (Nexavar)
Pazopanib (Votrient)
Crizotinib (Xalkori)
Vandetanib (Caprelsa)
Ruxolitinib (Jakafi)
Tofacitinib (Tasocitinib)

VEGF expression in hypoxic tumor areas (Liu et al. 2002). Inhibition of COX-2 activity reduces IL-6 and IL-8 secreted by tumor cells, prevent transformed cells from inducing suppression of cell mediated immune response and inhibits apoptosis (Tsujii et al. 1997; Taketo 1998; Subbaramaiah et al. 1997; Grinwick and Plescia 1977), supporting the link between inflammation and angiogenesis (Luca et al. 1997; Hong et al. 2000). Moreover, inhibition of COX-2 enhances radiosensitivity of malignant cells as well as decreasing the radiation-dependent effects of increased angiogenesis and normal cell toxicity (Choi and Milas 2003).

Several studies have demonstrated that regular use of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) is associated with a reduced risk of some cancers, in particular of the gastrointestinal tract, lung, breast cancer, and bladder cancer and also induce tumor regression (Brown and DuBois 2005; Thun and Namboodin 1991; Schreinemachers and Everson 1994; Funkhouser and Sharp 1995; Mann et al. 2005; Ulrich et al. 2006; Harris et al. 2006; Castelao et al. 2000; Lejeune et al. 2006). NSAIDs act as anti-angiogenic agents blocking the production of PGs, or through an inhibition of VEGF and HGF (Boccaccio et al. 2005).

The anti-inflammatory agent celecoxib (Celebra), originally approved by the FDA for the treatment of osteoarthritis, is now indicated also in the treatment of familiar adenomatous polyposis and in clinical trials for both prostate and pancreatic cancer for its anti-angiogenic activity (Demaria et al. 2010).

A biphosphonate compound, zoledronic acid suppressed MMP-9 expression by tumor associated macrophages and reduced the association of VEGF with its receptor on ECs (Giraudo et al. 2004). Scavelli et al. (2007) demonstrated tha zoledronic acid markedly inhibit in vitro proliferation, chemotaxis, and capillarogenesis of MMECs, and angiogenesis in vivo in the CAM assay (Fig. 5.1). Moreover, mevastatin, a specific inhibitor of the mevalonate pathway, reverts the zoledronic acid and anti-angiogenic effect, indicating that the drugs halts this pathway.

Rapamycin, an inhibitor of "mammalian target of rapamyicin" (mTOR), is a natural macrolide antibiotic, used for some years as immunosuppressive agent after organ transplantation (Motzer et al. 2008). Rapamycin is antiangiogenic (Guba

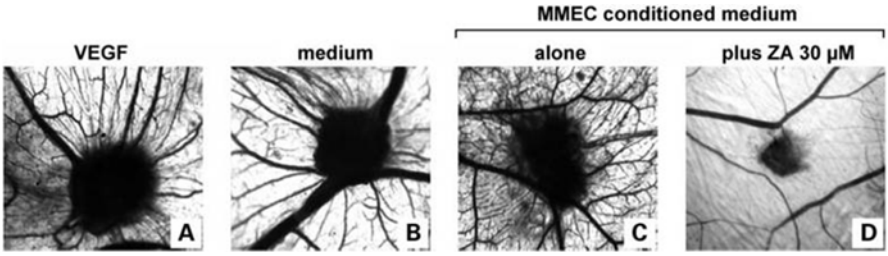

Fig. 5.1 CAMs treated with sponges loaded with VEGF165 or multiple myeloma endothelial cells (MMEC) conditioned media are surrounded by allantoic vessels developing radially toward the implant in a spoked-wheel pattern (**a** and **c**). No vascular response is detectable around the sponges loaded with vehicle alone (**b**). Zoledronic acid added to the conditioned media significantly inhibits the angiogenic response (**d**) (Reproduced from Scavelli et al. (2007))

et al. 2002) and inhibits endothelial cell cycle (Fig. 5.2) and primary and metastatic tumor growth (Marimpietri et al. 2005, 2007; Phung et al. 2007). Xue et al. (2009) examined the effects of rapamycin on VEGF-induced angiogenesis and lymphangiogenesis with an adenovirus expressing VEGF-164 in the ears of adult nude mice, and demonstrated that rapamycin inhibited the earliest stages of angiogenesis, including mother vessel formation and increased vascular permeability, while later stages formation and lymphangiogenesis were unaffected.

TNF-α promote angiogenesis and tumor growth by inducing a range of angiogenic factors, thymidine phosphorylase, and MMPs (Aggarwall 2003). TNF-α antagonists inhibit inflammatory cytokines, MMPs angiogenic activity and leukocyte trafficking to site of inflammation (Feldmann 2002). A number of clinical trials of TNF-α antagonists alone, and in combination with other therapies, are currently under way in cancer patients.

Blocking PlGF exert an anti-inflammatory activity, due to the fact that PlGF is a chemo attractant for VEGFR-1-positive macrophages (Luttun et al. 2002). Selective inhibition of platelet-endothelium interactions, using specific phenotypic characteristics of tumor endothelial cells as target, may be a potential therapeutic tool to reduce tumor angiogenesis. For example, selective stimulation of the platelet PAR-4 receptor may inhibit tumor angiogenesis by suppression of VEGF secretion and simultaneous enhancement of the release of endostatin.

Atiprimod has anti-inflammatory activities in animal models of rheumatoid arthritis and is well tolerated in phase I trials (Lakings 2003), inhibits the proliferation of MM cells lines *in vitro* by inhibiting signal transducer and activator of transcription 3 activation, thereby blocking the signaling pathway of IL-6, an angiogenic cytokine expressed in MM (Amit-Vazina et al. 2005).

Germano et al. (2013) demonstrated that trabectidin, an anti-cancer agent approved for late-stage soft-tissue sarcoma, exert anti-tumor responses by depleting monocytes and macrophages.

Therapeutic strategies may include inhibition of recruitment of mast cells and macrophages to the tumor microenvironment and blockade of pro-tumoral effects

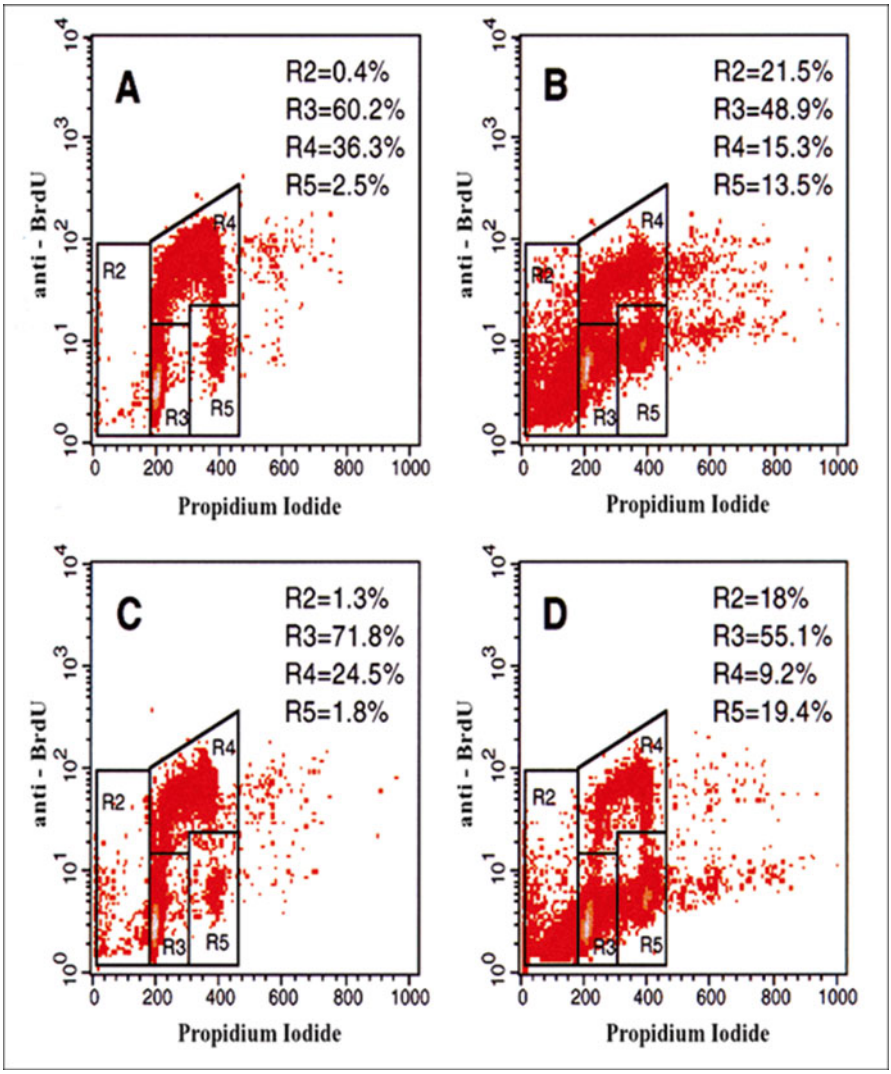

Fig. 5.2 Inhibition of endothelial cell cycle progression by vinblastine (*VBL*) and rapamycin (*RAP*). Endothelial cells were incubated for 48 h in the presence of complete medium (panel **a**), 50 pM VBL (panel **b**), 0.5 nM RAP (panel **c**) or 50 pM VBL plus 0.5 nM RAP (panel **d**). BrdU uptake (*FITC*) vs. total cellular DNA content (*PI*) was evaluated by densitometric FACS analysis. VBL increased the percentage of cells with G2-M and sub-G1 DNA content. RAP increased the G1 fraction suggesting inhibition of the cell cycle from G1 to S phase. VBL plus RAP decreased the percentage of cells in S phase (Reproduced from Marimpietri et al. (2005))

and pro-angiogenic functions. Mast cells might act as a new target for the adjuvant treatment of tumors through the selective inhibition of angiogenesis, tissue remodeling and tumor promoting molecules, allowing the secretion of cytotoxic cytokines and preventing mast cell mediated immune-suppression. Pre-clinical studies using anti-cKIT antibodies (Huang et al. 2008), anti-TNF-α antibodies (Gounaris et al. 2007), or the mast cells stabilizer disodium cromoglycate (cromolyn) (Soucek et al. 2007) in mouse models have demonstrated promising results.

Clodronate-liposomes depleted macrophages and inhibited tumor angiogenesis in mouse tumor transplantation models (Zeisberger et al. 2006). In the mouse cornea model, killing of COX-2 positive infiltrating macrophages with clodronate liposomes reduces IL-1-β-induced angiogenesis and partially inhibits VEGF-induced angiogenesis (Nakao et al. 2005). VEGF inhibitors decrease macrophage recruitment, and this effect may contribute to their anti-angiogenic activity (Giraudo et al. 2004). Specific inhibition of VEGFR-2 decreased tumor macrophage infiltration into orthotopic pancreatic tumors (Dineen et al. 2008). CSF-1 receptor (CSF1R) kinase inhibitors exhibit anti-angiogenic and anti-metastatic activity in tumors (Manthey et al. 2009). Anti-CSF-1 antibodies and antisense oligonucleotides suppress macrophage infiltration and xenograft tumor growth in mice (Aharinejad et al. 2002, 2004). Blockade of macrophage recruitment with CSF1R-signalling antagonists in combination with paclitaxel decreased vessel density, reduced tumor growth and pulmonary metastasis, and improved survival of mammary tumor-bearing mice (De Nardo et al. 2011). Trabectidin is a natural product derived from a marine organism with potent anti-tumor activity, cytotoxic for TAMs, and inhibits the production of CCL-2 and IL-6 (Germano et al. 2013).

In addition to its anti-angiogenic activity, thalidomide enhances T-cell- and NK-cell-mediated immunological responses, induces caspase-8 mediated apoptosis, and down-regulates IL-6 production within the BM microenvironment in MM (Davies et al. 2001; Mitsiades et al. 2002). A thalidomide analogue, lenalidomide, a COX-2 inhibitor and an anti-angiogenic agent, is currently used in the treatment of MM (Dimopoulos et al. 2007). Lenalidomide reduces the expression of VEGF and FGF-2 (Barlett et al. 2004). Lenalidomide exerts in vivo in the CAM assay a relevant anti-angiogenic effect, whereas in vitro lenalidomide inhibits MMECs proliferation and migration (De Luisi et al. 2011). Moreover, MMECs treated with lenalidomide show changes in VEGF/VEGFR-2 signaling pathway and in several proteins controlling EC motility, cytoskeleton remodeling, as well as energy metabolism pathways (Fig. 5.3) (De Luisi et al. 2011). Moreover, lenalidomide acts by disrupting the stroma support within the BM that is needed for the production of a range of cytokines. Lenalidomide inhibits VEGF-induced PI3K-Akt pathway signaling and HIF-1α expression (Lu et al. 2009), exerts an anti-TNF-α activity, modulates the immune response stimulating T cells and NK cells activities, induces apoptosis of tumor cells, and decreases the binding of MM cells to BM stromal cells (Mitsiades et al. 2002; Dredge et al. 2005; Hideshima et al. 2007; Görgön et al. 2010). A retrospective analysis of clinical trials, with previously treated relapsed/refractory MM, demonstrated an improved response rate and increased median for patients treated with lenalidomide and dexamethasone, compared to those treated

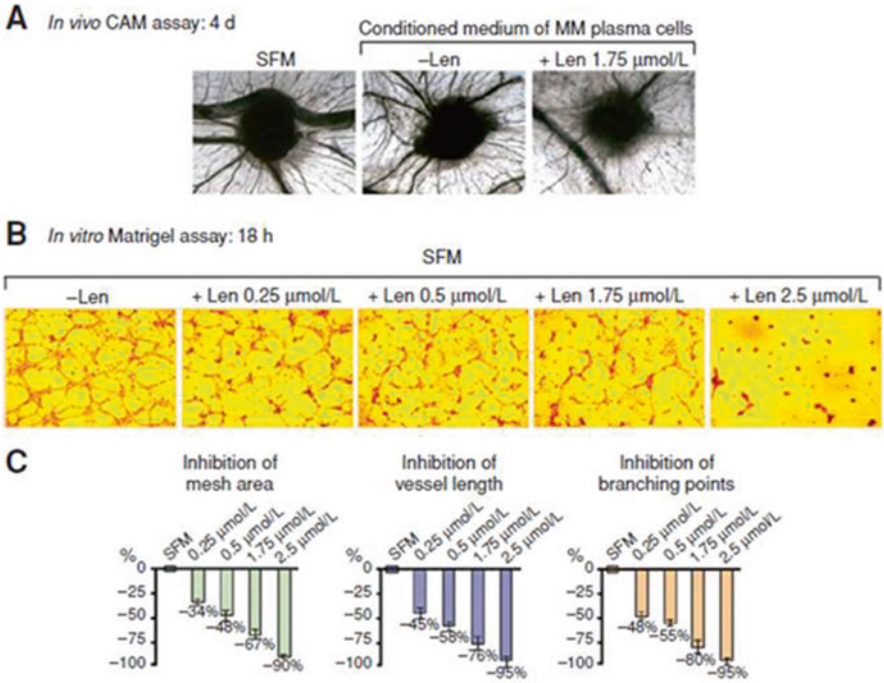

Fig. 5.3 Lenalidomide inhibits angiogenesis in CAM and Matrigel. (**a**) CAMs were incubated with gelatin sponges loaded with SFM (*left*) and with conditioned medium of multiple myeloma (*MM*) plasma cells either alone (*middle*) or supplemented with 1.75 mmol/L lenalidomide (*right*). Note the inhibition of MM angiogenesis by the drug. (**b**) lenalidomide inhibits MM endothelial cells (*MMEC*) angiogenesis in the Matrigel in a dose-dependent manner. MMECs arranged to form a closely knit capillary-like plexus (*left*), whereas the tube formation was gradually blocked with increasing lenalidomide doses with a full inhibition at 2.5 mmol/L (*right*). (**c**) skeletonization of the mesh was followed by measurements of its topological parameters: mesh area, vessel length, and branching points. Data are presented as mean ± SD of percent inhibition. *Len* lenalidomide (Reproduced from De Luisi et al. (2011))

only with dexamethasone (Wang et al. 2008; Dimopoulos et al. 2007; Weber et al. 2007). In a phase 2 study, lenalidomide/bortezomib/dexamethasone gave responses in 84 % of relapsed/refractory patients, including complete response or near complete response in 21 % (Dimopoulos et al. 2010), and produced responses in 98–100 % of newly-diagnosed patients (Barosi et al. 2012).

Bortezomib induces EC apoptosis (Williams et al. 2003), and inhibits VEGF, IL-6, Ang-1 and Ang-2 and IGF-1 secretion in BMSCs and ECs derived from patients (Fig. 5.4) (Hideshima et al. 2003; Roccaro et al. 2006). The use of bortezomib in pre-transplant induction therapy revealed a higher response rate, compared to other induction regimens (Rajkumar and Sonneveld 2009). Bortezomib and zoledronic acid inhibit macrophage proliferation, adhesion, migration, and expression of angiogenic cytokines and angiogenesis on Matrigel in MM patients. Moreover,

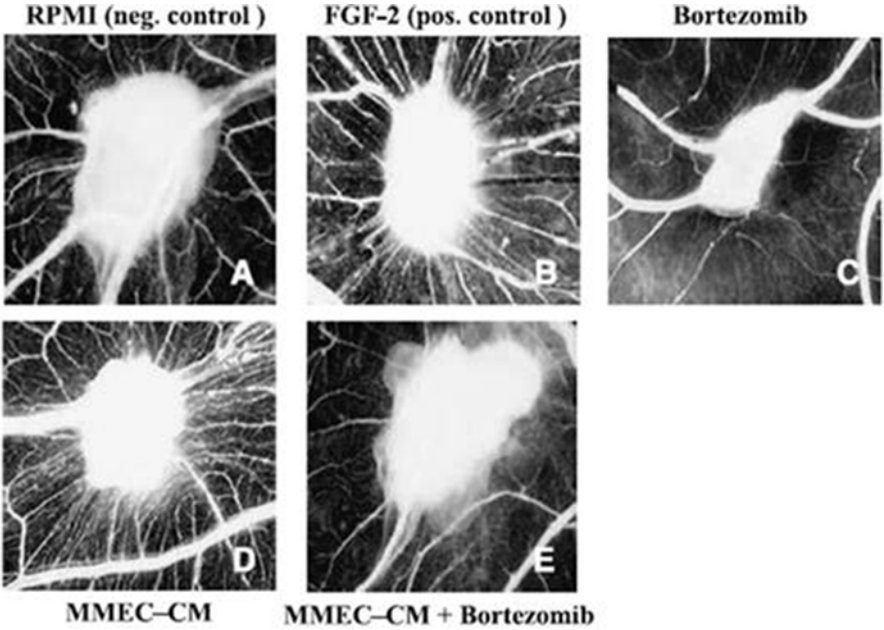

Fig. 5.4 Bortezomib inhibits angiogenesis in the CAM assay. The CAM was incubated with a gelatin sponge loaded with RPMI (**a**) 200 Ag/mL FGF-2 (**b**) 20 nmol/L bortezomib (**c**) and with MMECs conditioned media (CM) either alone (**d**) or with 20 nmol/L bortezomib (**e**). Bortezomib significantly inhibited basal angiogenesis induced by sponges loaded with vehicle alone (**c**). Moreover, CAM implanted with FGF-2 (**b**) or with MMECs CM (**d**) increased macroscopic vessel counts. Bortezomib significantly inhibited MMECs CM–induced angiogenesis (**e**), evidenced both by macroscopic vessel counts and by the number of eggs out of total with >50 % inhibition in the angiogenic response compared with vehicle (Reproduced from Roccaro et al. (2006))

VEGFR-2 and ERK1/2 phosphoactivation as well as NF-kB are also inhibited (Moschetta et al. 2010). Finally, bortezomib induces the differentiation of MSCs into osteoblasts and induces apoptosis of osteoclasts (Mukhejee et al. 2008; Chauhan et al. 2008).

The administration of inhibitors of osteoclasts activity, including bisphosphonates, not only prevents the MM-induced bone destruction, but also exerts an anti-angiogenic activity. Therapeutic doses of zoledronic acid markedly inhibit *in vitro* proliferation, chemotaxis and angiogenesis of MM endothelial cells and *in vivo* angiogenesis in the CAM assay (Scavelli et al. 2007). These data suggest that the zoledronic acid antitumoral activity in MM is also sustained by anti-angiogenesis, which would partly account for its therapeutic efficacy in MM (Henk et al. 2012; Morgan et al. 2012).

Vessel leakiness and chaotic blood flow may impair the bioavailability of chemotherapeutic drugs meant to target the tumor blood vessels and some of these morphological abnormalities may be reversible or normalized. The concept of

Table 5.4 Tumors with pre-clinical evidence of tumor vessel normalization upon anti-angiogenic treatment

Melanoma
Breast
Ovarian
Glioblastoma multiforme
Fibrosarcoma
Squamous carcinoma
No small cell lung cancer
Pancreatic
Liver fibrosarcoma
Insulinoma
Lung
Insulinoma
Vestibular schwannoma
Colon

"normalization" of tumor blood vessels by anti angiogenic drugs was introduced by Rakesh Jain in 2001. The state of normalization is probably transient, and dependent on the dose and duration of the treatment (Table 5.4).

VEGF inhibition could temporarily restore or normalize the function of tumor-associated vasculature, decreasing vascular permeability in conjunction with restoration of sustained pressure gradients, as demonstrated by intravital imaging studies in preclinical models and in cancer patients (Fukumura et al. 2010), thereby enhancing systemic delivery of oxygen or perfusion of cytotoxic agents to intratumoral sites (Tong et al. 2004; Jain 2005). Moreover, abrogation of VEGF signaling increases collagenase IV activity, leading to restoration of normal BM (Winkler et al. 2004), which generally in tumors has an abnormally thickness (Baluk et al. 2003).

Temporal vascular nomalization induces vascular regression, which causes tumor hypoxia (Sato 2011). Hypoxia could alter the property of cancer cells through the induction of HIF-1, as HIF-1 is reported to be involved in the induction of genes that induce invasive and metastatic properties of tumor cells (Semenza 2003). Hypoxia generated by angiogenesis inhibition triggers pathways that make tumors more aggressive and metastatic and less sensitive to anti-angiogenic treatment, as demonstrated by Paez-Ribes et al. (2009), who used blocking VEGFR-2 antibodies to mouse models of pancreatic neuroendocrine carcinoma and glioblastoma, and found that cancers showed heightened invasiveness or metastasis.

Helfrich et al. (2010) demonstrated in spontaneously developing melanomas of MT/ret transgenic mice after using anti-VEGF therapy and in human melanoma metastases taken at clinical relapse in patients undergoing adjuvant treatment with bevacizumab, that tumor vessels which are resistant to anti-VEGF therapy are characterized by enhanced vessel diameter and normalization of the vascular bed by coverage of mature pericytes.

VEGFR-2 blockade can lead to the upregualtion of Ang-1 that increase pericyte coverage of the vessels (Winkler et al. 2004). Ang-2 plays a more important role in

tumor angiogenesis than it does in normal angiogenesis, is responsible for blood vessel destabilization in vasculature surrounding tumors. In glioblastoma patients, the Ang-1/Ang-2 ratio correlates with survival (Sie et al. 2009) and vascular normalization, whereas high Ang-2 levels correlate with resistance to anti-VEGF therapy (Batchelor et al. 2010).

Blockade of VEGF signaling with the VEGFR tyrosine kinase inhibitor cediranib significantly reduced levels of Ang-2 in some patients, even if the decrease was transient and modest (Batchelor et al. 2010). Chae et al. (2010) expressed Ang-2 in an orthotopic glioma model and demonstrated that ectopic expression of Ang-2 had no effect on vascular permeability, tumor growth, or survival, but it resulted in higher vascular density, with dilated vessels and reduced mural cell coverage. When combined with anti-VEGFR-2 treatment, Ang-2 destabilized vessels and compromised the survival benefit of VEGFR-2 inhibition by increasing vascular permeability, suggesting that VEGFR-2 inhibition normalized tumor vasculature, whereas ectopic expression of Ang-2 diminished the beneficial effects of VEGFR-2 blockade by inhibiting vessel normalization.

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