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**INTERPLAY BETWEEN TUMOR ANGIOGENESIS
AND THE IMMUNE SYSTEM**
towards a new cancer therapy

Voor ons pap en ons mam
Voor Michel

INTERPLAY BETWEEN TUMOR ANGIOGENESIS AND THE IMMUNE SYSTEM towards a new cancer therapy

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. mr. G.P.M.F. Mols
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CHAPTER 1

**Interplay between
tumor angiogenesis and
leukocyte-vessel wall
interactions**

INTRODUCTION

The process of angiogenesis is involved in physiological situations such as the femal reproductive cycle¹, embryonic morphogenesis² and wound healing^{3,4}. Its primary function is to reverse tissue ischemia, accumulation of metabolic waste products and to supply tissues with nutrients. Excessive angiogenesis is usually associated with diseases such as diabetic retinopathy⁵, rheumatoid arthritis⁶, cardiovascular disease^{7,8} and cancer^{4,9}. Such diseases may benefit from therapeutic inhibition of angiogenesis¹⁰. It was already in the early 1970s that Folkman and colleagues launched the idea that tumors -because they are highly vascularized- are most vulnerable at the level of blood supply¹⁰. Research on angiogenesis inhibition in cancer really expanded in the 1990's after the discovery of the angiostatic compounds TNP-470 (AGM-1470) by Ingber and colleagues¹¹ and angiostatin by O'Reilly and colleagues¹². Since then, several potent angiogenesis inhibitors have been discovered, some of which are already being tested in clinical trials. Anti-angiogenesis therapy is now considered to be a promising approach for treatment of cancer and other pro-angiogenic diseases.

Once a tumor is vascularized it is not only accessible for plasma constituents, but also for blood cells. As a result, it can be infiltrated by leukocytes, a phenomenon observed in most solid cancers. The first observation of leukocyte infiltration in tumors was done by Virchow in 1863¹³. At that time, it was thought to be the result of a chronic inflammation that was already present before tumor development. Nowadays, however, it is known that the presence of leukocytes is a consequence of a specific immune reaction to the tumor itself, since the immune system is able to recognize various tumor-associated antigens¹⁴. Although associated with better prognosis, immune responses to a tumor are often weak and not able to completely destroy a tumor¹⁴, suggesting that tumors have developed mechanisms to escape immune surveillance. One mechanism to escape tumor-directed immunity is inhibition of leukocyte-vessel wall interactions in tumor vessels¹⁵⁻¹⁸, due to suppressed expression of endothelial adhesion molecules¹⁹⁻²² and endothelial cell anergy¹⁰. As a result, tumors frequently show only limited leukocyte infiltration²⁰.

In this thesis, focus will be on the interplay between tumor angiogenesis and the immune system. As an introduction, several aspects of tumor angiogenesis will be discussed in the first part of this chapter, with additional attention for the possible use of angiogenesis inhibition to improve cancer therapy. Subsequently, adhesion molecules involved in leukocyte-vessel wall interactions are described. In the next section, focus is on the effect of tumor angiogenesis on leukocyte-vessel wall interactions. Understanding behavior of leukocytes in tumor blood vessels may be of major impact for the improvement of immunotherapy strategies to cure cancer. In the last part of this chapter the aim and outline of this thesis are presented.

TUMOR ANGIOGENESIS

Tumor growth and angiogenesis

Without the formation of new blood vessels out of pre-existing ones (angiogenesis) the outgrowth of tumors is limited to 1-2 mm in size because of insufficient supply of oxygen and nutrients, a concept first proposed by Dr J. Folkman in 1971²³. Many tumors will not grow beyond this size and, therefore, stay clinically undetected for years²⁴. The process of angiogenesis is regulated by angiogenic and angiostatic factors. When the balance between these factors is in favor of the angiogenic ones, it may lead to the progression of tumors to an angiogenic phenotype, the so-called angiogenic switch^{25,26}.

The angiogenic cascade involves activation, migration and proliferation of vascular endothelial

Figure 1.1 The angiogenic cascade

After the angiogenic switch has occurred, a tumor starts to produce several angiogenic factors which bind to receptors present on nearby endothelial cells (ECs). Subsequently, the extracellular matrix (EM) is degraded by matrix metalloproteinases, allowing activated ECs to migrate in the direction of the stimulus. The ECs start to proliferate and form a lumen. Eventually, the newly formed vessel is matured due to the formation of an extracellular matrix and the presence of pericytes.

cells (ECs) and, eventually, vessel maturation (Figure 1.1). After a tumor has changed from a quiescent to an angiogenic phenotype in response to hypoxic or ischemic conditions, angiogenic factors are being produced which diffuse to nearby blood vessels and bind to counterreceptors on ECs. As a result, these ECs become activated and start to produce matrix metalloproteinases, which degrade the extracellular matrix. ECs are then able to migrate and proliferate in the direction of the tumor; ultimately, they will form a tube with a lumen. Vessel maturation is completed by recruitment of accessory cells and deposition of extracellular matrix, providing stabilization of the newly formed vessels^{4,8,10}. Besides allowing the tumor to grow, angiogenesis also provides a route for metastasing tumor cells to leave the tumor and enter the blood stream^{27,28}. On the other hand, the presence of blood vessels in a tumor also enables various immune cells to enter the tumor and perform their particular immune function. These immune cells may also secrete angiogenic and angiostatic factors (see chapter 5)²⁹.

Angiogenic factors in tumor formation

Among the known angiogenic growth factors involved in human cancer, the VEGF family appears to play a dominant role^{27,30}. VEGF, or vascular endothelial cell growth factor, is produced by hypoxic tumor cells and also by macrophages²⁷. The VEGF family consists of the prototype member VEGF

(also referred to as VEGF-A), placenta growth factor (PlGF), VEGF-B, VEGF-C and VEGF-D, in which the latter two are primarily associated with the formation of lymphatic vessels and lymphatic metastasis³¹. Alternative exon splicing results in the generation of four principal VEGF-A isoforms: VEGF¹²¹, VEGF¹⁶⁵, VEGF¹⁸⁹ and VEGF²⁰⁶, having 121, 165, 189 and 206 amino acids, respectively. VEGF¹⁶⁵, which is the predominant isoform, lacks the residues encoded by exon 6, while VEGF¹²¹ lacks those encoded by exons 6 and 7. Less frequent occurring splice variants have been reported as well, including VEGF¹⁴⁵, VEGF¹⁸³, VEGF¹⁶² and VEGF^{165b} (for further information see review by Ferrara et al.³²). From *in vitro* and *in vivo* studies it is known that VEGF promotes growth of vascular endothelial cells derived from arteries, veins and lymphatics^{32,33}. In addition to a stimulatory effect on every part of the process of angiogenesis, VEGF increases vascular permeability³⁴ and prevents upregulation of endothelial adhesion molecules induced by inflammatory cytokines¹⁹. *In vitro*, VEGF is known to prevent endothelial cell apoptosis, mediated by the phosphatidylinositol 3' kinase (PI3K)/Akt pathway and by induction of anti-apoptotic proteins in ECs³⁴. *In vivo*, the prosurvival effects of VEGF are mainly developmentally regulated: VEGF inhibition results in apoptotic changes in the vasculature of neonatal but not adult mice³⁵. Moreover, VEGF has been described to be a chemoattractant involved in monocyte recruitment to tumors via activation of VEGFR1^{27,36-38}. At present, two VEGF receptors are known: VEGFR1 (or Flt-1) and VEGFR2 (also known as Flk-1), which are expressed by ECs, tumor cells and monocytes/macrophages. VEGFR2 is considered to be the main mediator of the mitogenic, angiogenic and permeability enhancing effects of VEGF, while VEGFR1 does not seem to be directly involved in these processes. However, this receptor has been observed to be involved in the induction of matrix metalloproteinases and in the paracrine release of other endothelial cell derived growth factors³⁴. Both VEGF monoclonal antibodies and VEGF inhibitors are potentially interesting clinical tools in cancer.

Another family of angiogenic growth factors are the fibroblast growth factors, or FGFs. The most studied one is basic fibroblast growth factor (bFGF). This factor is secreted by macrophages, mast cells, lymphocytes, fibroblasts and by certain tumor cells³⁹⁻⁴¹. bFGF belongs to a large family of homologous polypeptide growth factors and promotes every phase of the angiogenic process, including synthesis of proteinases, EC migration and differentiated capillary tube formation *in vitro*³⁹. bFGF induces angiogenesis through the high-affinity heparin binding receptors FGFR-1 and FGFR-2⁴². Increased bFGF expression has been correlated with poor prognosis and bFGF expression by cancer cells shows a direct correlation with increased microvessel density⁴¹. bFGF is also able to induce angiogenesis indirectly by its capability to promote VEGF synthesis. In addition, there is a synergistic interaction between bFGF and VEGF in inducing EC proliferation and angiogenesis^{39,42}. Unfortunately, however, immunoneutralization of bFGF appears to induce little or no reduction in tumor angiogenesis^{43,44}, suggesting that other modulators of angiogenesis are more suitable targets for anti-angiogenesis treatment.

Besides VEGF and bFGF there are several other important factors that modulate angiogenesis, like tumor necrosis factor- α (TNF α), interleukin-8 (IL-8) and the matrix metalloproteinases (MMPs). TNF α is an inflammatory cytokine with angiogenic activity that is mainly expressed by tumor associated macrophages⁴⁵. Other sources are mast cells, T-lymphocytes, NK cells, neutrophils and some malignant cells⁴⁶. This cytokine has been found to promote the production of other angiogenic factors by tumor cells, like IL-8, bFGF and VEGF, through activation of transcription factors^{28,46}. IL-8 is a potent angiogenic factor in several cancers and associated with metastasis. It is reported to be produced by tumor cells, ECs, fibroblasts and macrophages⁴⁷. IL-8 expression significantly correlates with microvessel density⁴⁸, indicating the angiogenic potential of this interleukin. MMPs, released by both tumor cells and endothelial cells, are a family of zinc-dependent endopeptidases

which can cleave all major molecules of the extracellular matrix^{27,49,50}. During tumor growth, MMPs can enhance angiogenesis and degrade connective tissue and basement membranes to enable tumor growth and metastasis⁴⁹.

HIV-tat as an angiogenic factor

In patients infected with the human immunodeficiency virus-1 (HIV-1) the aortic endothelium is often severely damaged, which results in an increased risk of cardiovascular diseases and atherosclerosis. In addition, they frequently develop malignancies such as Kaposi's sarcoma. Kaposi's sarcoma is an angio-proliferative tumor, producing bFGF and VEGF, with prominent infiltration of monocytes and T-cells^{51,52}. The HIV-1 genome encodes several regulatory proteins that coordinate the expression, transport and assembly of virus particles, leading to virus production and transmission⁵¹. Among these regulatory proteins, HIV-tat appears to be essential for virus gene expression and replication⁵³⁻⁵⁵. The release of HIV-tat occurs already early after HIV-1 infection. However, it appears to be undetectable in chronically infected cells. During acute infection of T-cells by HIV-1, HIV-tat is released from these cells in an active form⁵⁵⁻⁵⁷. The tat-protein has been detected in sera of HIV-1-infected individuals⁵⁸ and in Kaposi Sarcoma lesions^{59,60}. One of the most striking properties of extracellular HIV-tat is its capability to mimic the effect of extracellular matrix proteins (such as fibronectin and vitronectin) on the locomotion, adhesion and growth of Kaposi Sarcoma cells and ECs, thereby enhancing angiogenesis and Kaposi Sarcoma progression⁵¹. Being considered as an angiogenic factor, HIV-tat differs from true angiogenic factors in that it can only act on ECs that are activated by inflammatory cytokines like IFN γ , TNF α and IL- β ^{1,51,61-64}. Furthermore, this protein is known to increase bFGF mediated angiogenesis and to bind VEGFR2⁵¹. The angiogenic properties of HIV-tat are mediated by three regions of the protein: the basic region that retrieves bFGF from heparan sulfate proteoglycans of the extracellular matrix (promoting EC growth)⁶⁵⁻⁶⁷, the cysteine-rich region which exerts a direct angiogenic effect by an unknown mechanism⁶⁸ and the RGD region that binds to the $\alpha 5 \beta_1$ and $\alpha v \beta_3$ integrins inducing EC migration⁶⁹.

INHIBITION OF ANGIOGENESIS TO INHIBIT TUMOR GROWTH

The newly formed blood vessels, which are essential for a tumor to grow and survive, can also be used by modern medicine to deliver anti-cancer drugs in efficient quantities to all regions of a tumor⁷⁰. In addition, the cells lining the luminal side of these blood vessels, the tumor ECs, are considered to be interesting targets for the delivery of certain agents, like inhibitors of angiogenesis, in order to modify their function.

Angiostatic agents work through various mechanisms, like inhibition of EC proliferation and migration, induction of EC apoptosis, or interference with MMP activity. It has been shown that when vessel formation is impaired or ECs are induced to undergo apoptosis, resulting in impaired oxygen and nutrient supply, tumor cells become apoptotic as well. At the moment, a variety of angiostatic agents has been discovered and developed, and numerous pre-clinical experiments involving angiogenesis inhibition are performed. There are also some chemotherapeutic agents known with claimed angiostatic properties, of which these angiostatic effects in animal models of cancer are tested. Moreover, clinical trials with human subjects using either direct or indirect angiostatic agents are carried out. Examples of such agents and their effects are discussed below.

One method to interfere with angiogenesis is the blockage of angiogenic factors like VEGF or FGFs and their receptors. This can be performed by treatment with specific antibodies against these factors and their receptors, with soluble receptors functioning as antagonists or VEGF antisense

constructs¹⁰. Tumor growth inhibition by VEGF neutralization has been demonstrated by laboratories using several anti-VEGF approaches. At present, the furthest clinically developed VEGF inhibitor is bevacizumab (Avastin, developed by Genentech)³⁴. This agent is a humanized anti-VEGF monoclonal antibody, neutralizing all VEGF-A isoforms but not other members of the VEGF family. For the treatment of metastatic colorectal cancer, the addition of bevacizumab to chemotherapy results in improved overall survival and improved progression-free survival. The role of this agent in other tumor types is currently under investigation, including phase III clinical trials in non-small-cell lung cancer (NSCLC), renal-cell cancer and metastatic breast cancer³⁴. Another way to interfere with VEGF signalling is through VEGF receptor blockade by tyrosine kinase inhibitors like SU5416 (Semaxinib), SU6668 and SU11248 (all developed by Sugen), ZD4190 and ZD6474 (both developed AstraZeneca) and PTK787 (developed by Novartis). In spite of earlier encouraging results, the VEGFR-2 blocking agent SU5416 failed to demonstrate efficacy in a controlled phase III trial in colorectal cancer patients in combination with chemotherapy^{34,71}. SU6668 (blocking VEGF, FGF and PDGF receptors) and SU11248 (an oral multitargeted tyrosine kinase inhibitor with antitumor and anti-angiogenic activity through targeting, amongst others, VEGF and PDGF receptors) are both being investigated in clinical trials^{71,72}. ZD4190 and ZD6474 are both VEGFR-2 blocking agents, being in phase I and II respectively⁷¹. PTK787, a receptor tyrosine kinase acting on both VEGFR-1 and VEGFR-2, is in phase III clinical trials for colorectal cancer and is also in phase II clinical trials for a number of other tumors^{34,72}. An alternative way to neutralize VEGF is represented by the VEGF-trap, soluble receptors developed by Regeneron which is currently in phase I/II trials.

One of the first alternative compounds with angiostatic properties studied was AGM-1470 or TNP-470. TNP-470 is a synthetic analogue of fumagillin, that blocks the growth of new blood vessels by inhibiting methionine aminopeptidase, an enzyme that plays a key role in EC proliferation¹¹. Preclinical studies support a role for TNP-470 in slowing tumor growth, although it does not seem able to shrink existing tumors⁷³. Combination of TNP-470 with paclitaxel results in tumor regression, disease stabilization and improved survival⁷⁴. TNP-470 combined with paclitaxel and carboplatin was recently evaluated in the phase I setting in several tumor types⁷⁵. Overall, this regimen was well tolerated and resulted in a partial response in 24% of the patients and a stable disease in 47%. In conclusion, TNP-470, preferably in combination with other drugs, may prove to be a promising tool in the treatment of cancer.

Angiostatin is a potent endogenous angiogenesis inhibitor identified by O'Reilly et al¹² in Lewis lung carcinoma models. This agent is a cleavage product of plasminogen, which has been shown to inhibit EC proliferation and impairs both primary and metastatic tumor growth through inhibition of angiogenesis⁵⁰. It was shown in animal models that continuous exposure to angiostatin is more effective in inhibiting tumor growth than bolus injections⁷⁶. For clinical purposes, recombinant angiostatin was developed by Entremed and given to patients in a twice daily schedule (mimicking the pharmacological profile of continuous infusion)⁷⁷. Treatment with recombinant angiostatin appears to be safe and feasible even after prolonged use (> 6 months). However, none of the patients included in this clinical trial had obvious tumor responses; in only one patient 30% reduction of tumor volume was observed.

Endostatin, a fragment of collagen XVIII, is an endogenous angiostatic factor (like angiostatin), inhibiting EC proliferation and increasing the rate of apoptosis in tumor cells⁷⁸. It has been shown to reduce tumor growth and even to induce nearly complete regression of established tumors in murine Lewis lung cancer models⁷⁸. The antitumor effects of both endostatin and angiostatin are associated with a downregulation of vascular endothelial growth factor (VEGF) expression by tumor cells⁷⁹. Recently, endostatin has entered phase I clinical trials^{80,81}, from which it is known to be

well tolerated. Although significant death of tumor ECs and reduced microvessel density were observed in response to a dose response curve, endostatin failed to induce high levels of tumor cell death resulting in only minor anti-tumor activities⁸². Further studies are needed to investigate the effects of endostatin on survival.

In search of novel angiostatic molecules, we have designed a β -sheet forming peptide named anginex. This peptide is designed by using a combination approach employing basic folding principles and incorporating short sequences from the β -sheet domains of angiostatic proteins like endostatin, platelet factor-4 (PF4), tumor necrosis factor- α (TNF α) and bactericidal permeability-increasing (BPI) protein⁸³. Anginex has been shown to be a potent inhibitor of angiogenesis by blocking EC proliferation, adhesion and migration and by inducing EC apoptosis⁸³. Moreover, we have shown that anginex is capable of angiogenesis inhibition in an *In vivo* tumor model, resulting in reduced tumor proliferation as compared to untreated mice^{83,84}. The current results indicate that anginex is a powerful anti-tumor angiogenesis inhibitor that shows promise of being developed to treat human cancers.

Conventional cytotoxic chemotherapeutics have been developed for the treatment of cancer by directly killing the tumor cells or inhibiting their proliferation. Several of these agents have been shown to possess angiostatic activity as well, presumably due to the presence of dividing ECs in newly forming tumor blood vessels⁸⁵. Maximal angiostatic activity typically requires prolonged exposure to low drug concentrations, using frequent schedules at doses substantially lower than the maximum tolerated dose (metronomic dosing)⁸⁶. Paclitaxel (Taxol) is one such agent of which the cytotoxic activities as a microtubule-stabilizing antineoplastic drug are well known. In addition, paclitaxel has been described to possess angiostatic activities, inhibiting EC proliferation⁸⁷, motility, invasiveness, and cord formation on Matrigel in a dose-dependent manner⁸⁸. In clinical trials, paclitaxel showed to be effective against several tumors, including ovarian, breast, lung, head and neck carcinomas⁸⁹. At the moment, the angiostatic properties of this agent are being tested in phase III trials where optimal dosing and treatment strategies are being adjusted^{90,91}.

Cyclophosphamide is a widely-used cytotoxic chemotherapeutic drug in cancer therapy that has been described to inhibit EC proliferation and, hence, angiogenesis as well⁸⁷. When cyclophosphamide was administrated according to a low-dose, frequent schedule, it decreased tumor volumes⁹² and increased apoptosis of tumor ECs and tumor cells were observed in several tumor models⁹³. These angiostatic effects observed with low doses of cyclophosphamide, or agents like paclitaxel, may be due to induction of thrombospondin-1, a potent endogenous and endothelial specific inhibitor of angiogenesis⁸⁵. In a pre-clinical setting, a combination of cyclophosphamide and TNP-470 even eradicated drug-resistant Lewis lung carcinomas⁹³. Thus far, only few clinical trials have directly tested angiostatic schedules of chemotherapy⁸⁶, so further clinical studies are needed to exploit the interesting field of chemotherapeutic drugs and anti-angiogenesis.

LEUKOCYTE-VESSEL WALL INTERACTIONS

Immune responses can be classified as acquired (adoptive) or innate (non-adoptive) immunity. Both types of immune responses are carried out by leukocytes, which can be divided in two major groups: polymorphonuclear (PMN) cells or granulocytes, and monomorphonuclear (MMN) cells. PMNs can be divided in neutrophils, eosinophils and basophils and are part of the innate immune system, since they use non-specific mechanisms to recognise and destroy pathogens. Monocytes, lymphocytes and natural killer (NK) cells belong to the MMNs. Monocytes and NK cells are also part of the innate immunity, whereas lymphocytes are involved in acquired immunity, generating a specific

immune response against individual pathogens⁹⁴.

In order to obtain a proper inflammatory response it is necessary to recruit leukocytes from the circulation into infected extravascular locations. This recruitment process is complex and involves interactions between leukocytes and ECs⁹⁵. The migration of leukocytes from the vasculature into the extravascular tissue occurs in several steps, as illustrated in Figure 1.2: capture, rolling, activation, firm adhesion, diapedesis and migration⁹⁶. This multistep cascade, in which every step needs to be completed for the next step to occur, depends on cell adhesion molecules expressed by both ECs and leukocytes, under tight control of the local microenvironment⁹⁷. First, the endothelium is activated by substances released at a site of inflammation or by a tumor. As a result, ECs start to express adhesion molecules which can weakly bind margined leukocytes. These captured leukocytes may start rolling along the vessel wall. Because of their low velocity and, hence, longer transit times, rolling leukocytes can become activated by chemotactic cytokines as well. These activated leukocytes will then spread and firmly adhere to the endothelium. Finally, they may migrate through the vessel wall (diapedesis) into the underlying tissue.

The first contact of margined leukocytes with activated endothelium is referred to as leukocyte capture or tethering. This is a weak, reversible binding and leukocytes can be observed 'jumping' along the endothelium. Once leukocytes are captured, they may start to roll along the vascular endothelium. Rolling occurs at a velocity significantly lower than that of the free flowing cells in the same blood vessel. The weak binding of rolling leukocytes to the endothelium is reversible

Figure 1.2 Leukocyte-vessel wall interactions

When leukocytes marginate from the blood stream in the direction of the vessel wall, they can be captured and may start rolling along activated endothelial cells (ECs) that express adhesion molecules. During the process of rolling, leukocytes may become activated as well, resulting in increased expression and binding affinity of integrins. As a result, they can firmly adhere to the endothelium and migrate through the vessel wall (diapedesis) into the surrounding tissue. EM: Extracellular Matrix.

as well and, hence, leukocytes can detach again. Both leukocyte capture and rolling are mediated by adhesion molecules of the selectin family that bind to glycoprotein ligands (Table 1.1)^{98,99}.

Table 1.1: Selectins and their counterreceptors involved in leukocyte rolling

Selectins	Expression	Ligands
L-selectin	granulocytes, monocytes, most lymphocyte subsets	PSGL-1, PNAd, P-selectin, E-selectin, MadCAM-1, unknown
P-selectin	activated ECs, activated platelets	PSGL-1, L-selectin, unknown
E-selectin	activated ECs	PSGL-1, L-selectin, unknown

Three selectin family members are known: L-selectin on Leukocytes, P-selectin on Platelets and ECs, and E-selectin on ECs. L-selectin is constitutively expressed on all granulocytes and monocytes, and on most lymphocytes. P-selectin is stored in α -granules of platelets and in Weibel-Palade bodies of ECs; it is quickly translocated to the cell surface after activation⁹⁸. E-selectin is not stored in granules but requires de novo mRNA and protein synthesis to be expressed on the surface of activated ECs⁹⁸. In non-inflamed skin microvessels, P- and E-selectin are constitutively expressed on ECs^{100,101}. P-selectin glycoprotein ligand (PSGL-1) is a ligand for all three types of selectins and is expressed by neutrophils, lymphocytes and monocytes¹⁰². PSGL-1 accounts for about 90% of P-selectin binding and is also the most important counterreceptor for L-selectin. In addition, both E- and P-selectin are known to bind L-selectin. Other L-selectin ligands have been identified in high endothelial venules and are collectively known as peripheral node addressins (PNAd)⁹⁸. Although PSGL-1 is able to bind E-selectin as well, the major E-selectin ligand remains poorly defined¹⁰². The crucial role of selectins in leukocyte recruitment is demonstrated in mice that lack selectins or PSGL-1¹⁰³, and in patients suffering from a rare disease called leukocyte adhesion deficiency type II (LAD-II). These individuals are not capable to effectively incorporate fucose into their selectin ligands; as a result, these ligands fail to bind L-, P-, and E-selectin. These patients frequently have bacterial infections and are treated with oral fucose and/or antibiotics⁹⁸.

The adhesion molecules on leukocytes that are involved in adhesion and diapedesis are usually present on the cell surface or have to be released from intracellular granules. These so-called integrins can be upregulated through an increase in the number of molecules expressed on the cell surface, while their activity can be enhanced through conformational changes that increase their binding affinity. For this purpose, leukocytes need to become activated by chemotactic cytokines or chemokines¹⁰⁴ like, for instance, complement factor 5a, TNF α , lipopolysaccharide (LPS), interleukin-8 (IL-8), RANTES and leukotriene-B₄ (LTB₄)⁹⁴. Upon activation, the expression and affinity of these integrins are greatly enhanced. Integrins consist of two non-covalently bound polypeptides (α and β chains), traversing the cell membrane. β_1 -integrins, like the very late antigens (VLA-4 and VLA-5), are mainly involved in binding of leukocytes to the extracellular matrix⁹⁴. Integrins with a β_2 subunit, like lymphocyte function-related antigen (LFA-1) and membrane attack complex-1 (Mac-1), are only expressed by leukocytes and involved in leukocyte-endothelium interactions. In patients with leukocyte adhesion deficiency type I (LAD-I), recurrent, life threatening bacterial infections are seen due to the absence of β_2 -integrins¹⁰⁵. Patients with LAD-I fail to recruit leukocytes, indicating the importance of the integrin family in immunity. A number of integrins relevant for leukocyte adhesion to the endothelium are summed in Table 1.2.

The endothelial counterreceptors for the integrins are members of the immunoglobulin gene

Table 1.2: Integrins and their counterreceptors involved in leukocyte adhesion

Integrins	Expression	Ligand(s)
VLA-4 ($\alpha 4,1$)	lymphocytes, some phagocytes	VCAM-1, fibronectin
VLA-5 ($\alpha 5,1$)	leukocytes, platelets	Fibronectin
LFA-1 ($\alpha L,2$)	most leukocytes	ICAM-1, ICAM-2
Mac-1 ($\alpha M,2$)	granulocytes, phagocytes	ICAM-1, ICAM-2

superfamily, which consists of glycoproteins with multiple immunoglobulin-like domains. These proteins are expressed by vascular ECs, leukocytes and platelets. The intercellular adhesion molecules (ICAM-1 and ICAM-2) and vascular cell adhesion molecule (VCAM-1) are examples that are involved in leukocyte adhesion. Platelet endothelial cell adhesion molecule (PECAM) and mucosal addressin cell adhesion molecule-1 (MadCAM-1) are members of the same family⁹⁴ (Table 1.3).

Table 1.3: Immunoglobulin gene superfamily members and their counterreceptors involved in leukocyte adhesion and diapedesis

Immunoglobulin gene superfamily	Expression	Ligand(s)
ICAM-1	ECs, leukocytes	LFA-1, Mac-1
ICAM-2	ECs, leukocytes	LFA-1, Mac-1
VCAM-1	activated ECs, macrophages	VLA-4
PECAM-1	leukocytes, ECs, platelets	PECAM-1
MadCAM-1	high endothelial cells	L-selectin

Following leukocyte adhesion diapedesis may occur, after which leukocytes can migrate into the surrounding (tumor) tissue. Diapedesis occurs within a few minutes during which the leukocyte extends itself by forming pseudopodia across the endothelial border¹⁰⁶. For diapedesis to occur, adhesion molecules of the integrin and the immunoglobulin gene superfamily are needed, like PECAM-1 (CD31) and CD99^{96,106}. Furthermore, this process requires an increase in intracellular free calcium in ECs that are adjacent to a transmigrating leukocyte. The increase in intracellular free calcium activates myosin light chain kinase which leads to unfolding of myosin II, leading to EC retraction that might facilitate leukocyte passage¹⁰⁶.

Once leukocytes have transmigrated from the blood stream into the tissue, they can migrate through the tissue in the direction of the stimulus, like a site of inflammation or a tumor, and exert their local effects. Under normal physiological circumstances, leukocytes are recruited to a site of inflammation by the local synthesis of chemokines (chemoattractive cytokines) and cytokines and by products of tissue breakdown¹⁰⁶. In tumors, similar chemoattractive factors and adhesion molecules are thought to play a role in leukocyte recruitment. In contrast to cells at a site of inflammation, tumor cells do not stop proliferating and migrating and they continue to send out signals that recruit leukocytes to the tumor site¹⁰⁷. This concept has contributed to the characterization of tumors as “wounds that fail to heal”¹⁰⁸.

TUMOR ANGIOGENESIS AND THE IMMUNE SYSTEM

It is currently well established that tumor angiogenesis and the immune system do influence each other. On the one hand, components of the immune system appear to be involved in the regulation of tumor angiogenesis. A number of studies show that several leukocyte subsets can induce blood vessel formation in tumors. Examples are polymorphonuclear (PMN) granulocytes that produce matrix metalloproteinases (MMPs), VEGF and IL-8^{14,107}, CD4+ and CD8+ T-lymphocytes that produce VEGF and bFGF^{109,110}, mast cells that produce MMP9, VEGF, IL-8, bFGF, TNF α ^{107,111} and monocytes/macrophages producing e.g. VEGF, IL-8, bFGF, TNF α , IL-1 and MMP-9¹¹² (for further elaboration see chapter 5). On the other hand, angiogenic processes can have a major impact on immune cells and the development of an immune response¹⁰. As depicted in Figure 1.2, ECs are involved in leukocyte recruitment to sites of inflammation by the expression of adhesion molecules. In tumors, the process of angiogenesis increases the EC surface, enhancing the possibilities for leukocytes to interact with the vessel wall. Interestingly, however, relatively low levels of leukocyte-vessel wall interactions are being observed in tumors (see chapter 2)^{10,19,22,113,114}, which may be considered as a tumor-evolved mechanism to escape immune surveillance. It has been shown that exposure of ECs to angiogenic factors (that are produced by cancer cells, such as VEGF and bFGF) downregulates adhesion molecule expression^{115,116} and, hence, leukocyte-vessel wall interactions *in vitro*¹¹⁷ and also *In vivo*¹¹⁸. Moreover, an angiogenic environment hampers the upregulation of adhesion molecule expression by proinflammatory cytokines, a phenomenon called endothelial cell anergy¹¹⁹. Angiogenesis inhibition, either by specific angiostatic compounds or by chemotherapeutic agents with claimed angiogenesis inhibitory activity, may be a way to increase leukocyte-vessel wall interactions and subsequent infiltration in tumors (see chapter 3) and may be applicable for the improvement of various immunotherapeutic approaches²⁹.

AIM AND OUTLINE OF THE THESIS

In European and American countries, cancer is one of the most important causes of death. For the treatment of cancer, it is necessary to fundamentally understand the process of angiogenesis and its effects on the immune system. The aim of the present study was to investigate the relationship between angiogenesis and immunity in tumors and to explore ways to modulate this relationship in order to contribute to the development of new anticancer therapies. As presented in this thesis, we have developed a new tumor model in which both angiogenesis and leukocyte-vessel wall interactions in- and outside a tumor can be quantified noninvasively, allowing longitudinal analysis during tumor development and/or in the course of anticancer treatment. Using this tumor model, focus will be on leukocyte-vessel wall interactions in vessels both in- and outside a tumor as well as in healthy venules. Furthermore, an attempt was made to influence leukocyte interactions with the vessel wall by using both inhibitors and stimulators of angiogenesis. Understanding the behavior of leukocytes in tumor blood vessels may be of major impact when improving immunotherapy strategies in order to cure cancer.

The first experimental study, described in chapter 2, focuses on the development of a noninvasive method to investigate leukocyte-vessel wall interactions in both healthy and tumor vessels. By injecting a dense tumor cell suspension in mouse ear skin or on the flank we grew a small or a large tumor, respectively. The transparent mouse ear was chosen, since it allows intravital microscopic measurements on leukocyte-vessel wall interactions and local fluid dynamic conditions. In addition, these measurements can be performed non-invasively, allowing longitudinal analysis during tumor development and/or course of cancer treatment. Moreover, we performed *ex vivo*

and *in vitro* experiments to detect the expression of adhesion molecules on both freshly isolated and cultured endothelial cells using flow cytometry. We observed a significantly decreased level of leukocyte-vessel wall interactions in tumor vessels due to a decreased expression of adhesion molecules on tumor endothelial cells in response to angiogenic factors. This observation was identified as a tumor derived mechanism to escape immune surveillance.

Since we hypothesize that immune infiltration is detrimental to a tumor, we tried to upregulate leukocyte-vessel wall interactions in tumor vessels by antiangiogenic therapy in the next study (chapter 3). We used several specific inhibitors of angiogenesis (the new designer peptide anginex, endostatin, angiostatin and TNP-470) and two antineoplastic drugs (paclitaxel and cyclophosphamide) with claimed angiostatic properties. Using some of these compounds we indeed observed an upregulation in the number of interacting leukocytes in tumor vessels. Moreover, we showed that this increase in leukocyte-vessel wall interactions was due to an increase in endothelial cell adhesion molecule expression by antiangiogenic therapy. As a result we observed an increased leukocyte infiltration in tumors, presumably leading to increased anti-cancer activity.

In the study described in chapter 4, we investigated the influence of human immunodeficiency virus-1 (HIV-1) infection on angiogenesis and the development of angioproliferative lesions such as Kaposi's sarcoma. HIV-tat, the transactivator protein of HIV-1 released by infected cells, was found to increase leukocyte-endothelium interactions both *in vitro* and *In vivo* (alone and synergistically with TNF α), indicating that these proteins may contribute to the prominent infiltration of monocytes and T-cells in Kaposi's sarcoma.

In the chapters 2-4, we describe the effects of the process of angiogenesis and angiogenic factors on the function of the immune system. In chapter 5 we review the effects of the immune system (in this case monocytes/macrophages specifically) on the process of angiogenesis. Some tumors have been observed to contain a high number of infiltrated monocytes/macrophages, leading to a worse prognosis for the patient. In this chapter we discuss the pro- and anti-angiogenic factors produced by these immune cells and their effects on tumor angiogenesis, proliferation and metastasis.

Chapter 6 is a general discussion, in which the findings of the various chapters are discussed in an integral way, with suggestions for future investigation.

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CHAPTER 2

Tumor angiogenesis modulates leukocyte-vessel wall interactions *in vivo* by reducing endothelial adhesion molecule expression

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ABSTRACT

The expression of endothelial cell (EC) adhesion molecules involved in leukocyte-vessel wall interactions is suppressed in malignancies. In the present study, we investigated *in vivo* the regulation of leukocyte-vessel wall interactions by the presence of a tumor. By means of intravital microscopy, TNF α -stimulated leukocyte-vessel wall interactions were studied in ear skin microvessels of nude mice bearing small human LS174T colon carcinomas and in C57Bl/6 mice bearing murine B16F10 melanomas. Leukocyte-vessel wall interactions were studied both within and outside small tumors growing in the ear, and in ear microvessels of mice with a large tumor growing on their flank. Tumor-free mice were used as controls. Compared to values measured at the edge of the ear and in the contralateral ear, leukocyte adhesion was found to be significantly diminished in vessels inside the ear tumor in both mouse models. This reduction disappeared with increasing distance from the tumor. Surprisingly, the level of leukocyte adhesion in ear venules of mice with a large flank tumor was also significantly reduced. Leukocyte rolling, i.e. the step preceding adhesion, was not influenced by the presence of a tumor in nude mice, but was downregulated in immune competent C57Bl/6 mice. Treatment of mice bearing a small ear tumor with a humanized anti-VEGF antibody prevented the downregulation of leukocyte-vessel wall interactions inside the tumor vessels compared to the non-treated group. FACS-analysis showed that isolated tumor endothelial cells (ECs) have suppressed levels of ICAM-1 as compared to ECs from normal mouse tissues. In cultured b.END5 cells the TNF α -induced upregulation of ICAM-1 and VCAM-1 was reduced in ECs that were pre-incubated with bFGF or VEGF. The current results may have an impact on the effectiveness of clinical immunotherapeutic treatment protocols, because immune effector cells may not be able to enter tumor tissue.

INTRODUCTION

It has been known for some time that both immunotherapy¹ and inflammation² can contribute to the regression of solid tumors. To infiltrate tissues, including tumors, leukocytes have to interact with the venular vessel wall. These interactions start with leukocytes becoming tethered to and then slowly rolling along the vascular wall. This may result in firm adhesion with subsequent activation of the leukocytes, followed by their diapedesis and emigration into the surrounding tissue³. ECs can influence the selection of leukocyte subtypes that compose the immune infiltrate, by regulating adhesion molecule expression. By interfering with this regulated expression, tumors may have developed a mechanism of escaping from immune infiltration^{4,7}.

Angiogenesis, the formation of new blood vessels from pre-existing ones, is a prerequisite for outgrowth and metastasis of tumors^{8,9}. To achieve this, tumors secrete high levels of angiogenic factors such as vascular endothelial cell growth factors (VEGF) and fibroblast growth factors (FGF). Previously, we have shown that these factors are responsible for downregulating endothelial adhesion molecules such as intercellular adhesion molecule-1/-2 (ICAM-1/-2) and CD34^{4,10}. Angiogenic factors also inhibit inflammatory cytokine induced expression of vascular cell adhesion molecule-1 (VCAM-1) and E-selectin^{11,12}. In addition, it has been established both *in vitro*¹³ and *in vivo*¹⁴ that suppressed adhesion molecule expression by angiogenic factors results in diminished leukocyte-vessel wall interactions.

The present study aimed to investigate the regulation of adhesion molecule expression and leukocyte-vessel wall interactions *in vivo* by the presence of a tumor. To this end, a mouse model was developed that allows the investigation of leukocyte-vessel wall interactions, both inside and outside a tumor. The effects of a small tumor in the ear were compared with those of a larger tumor on the flank. Intravital microscopy was used to quantify the effects of the tumor on leukocyte-vessel wall interactions and on local fluid dynamic conditions in ear skin microvessels. To this purpose, a xenograft model was used, in which a human tumor (LS174T) was grown in nude mice. These experiments were repeated using immune competent mice (C57Bl/6) with a mouse tumor (B16F10). In addition, *ex vivo* and *in vitro* experiments were performed in order to detect ICAM-1 and VCAM-1 expression on ECs isolated from both tumor and healthy tissues (obtained from Swiss/nude mice and C57Bl/6 mice) and on mouse b.END5 brain endothelioma cells using flow cytometry.

MATERIALS AND METHODS

Animals

The experiments were approved by the local Ethical Review Committee on Animal Experiments. We used Swiss/nude mice (23-31 g, Charles River, Maastricht, the Netherlands), housed under sterile conditions, and C57Bl/6 mice (25-30 g, Charles River), housed under standard conditions.

Cell culture

Tumor cells (LS174T) were cultured in Dulbecco's MEM (Life Technologies, Paisley, Scotland) containing 10% inactivated fetal calf serum (FCS, Bio Whittaker, Verviers, Belgium), glutamine and antibiotics, and harvested using 0.25% trypsin. Mouse B16F10 melanoma cells (kindly provided by dr. J. Fidler, Houston, Texas) were cultured similarly using Hank's MEM (Life Technologies) containing 5% FCS and antibiotics, and harvested using 0.25% trypsin. Mouse b.END5 brain endothelioma cells (ECACC, Salisbury, United Kingdom) were cultured in Dulbecco's MEM (Life Technologies) containing 10% FCS.

Experimental protocol and intravital microscopy

In vivo experiments were performed in 4 groups of Swiss/nude mice. After anesthetizing these mice briefly (i.p.) using a mixture of ketamine (0.1 mg/g body weight (b.w.) Nimatek, Ad Usem Veterinarium, Cuijk, the Netherlands) and xylazine (0.05 mg/g b.w. in nude mice and 0.02 mg/g b.w. in C57Bl/6 mice, Sedamun, Ad Usem Veterinarium), the first group (ear tumor group; $n=6$) received 10 μ l of a dense tumor cell suspension (10^6 cells) intradermally in the dorsal side of the left ear. Within 3-5 days the ear tumor grew to a diameter of about 2 mm, at which time point the experiment was performed. In the second group (flank tumor group; $n=4$) 100 μ l of a tumor cell suspension (10^6 cells) was injected subcutaneously into the flank. In this group experiments were performed after 2-3 weeks, at which time the flank tumor had reached a diameter of about 1.5 cm. The third group served as control ($n=4$). These mice were not injected with tumor cells, but were otherwise treated similarly. Two additional mice bearing a small LS174T ear tumor ($n=2$) were treated with HuMV833 (a humanized anti-VEGF antibody, 3.0 mg/kg, i.p.; kindly provided by Protein Design Labs, Fremont, USA) on the 5th and 7th day after injection of the LS174T cells in the left ear. On day 8, intravital microscopy was performed. The experiments were repeated with three groups of C57B/6 mice carrying B16F10 murine melanoma tumors: an ear tumor group ($n=7$), a flank tumor group ($n=4$) and a control group ($n=4$).

Four hours prior to the start of the intravital microscopic observations, Swiss/nude mice received 750 ng recombinant murine TNF α (R&D Systems, Abingdon, U.K.) while C57Bl/6 mice received 500 ng TNF α . Two additional Swiss/nude mice as well as two C57Bl/6 mice were investigated that did not receive TNF α , to serve as control for the TNF α effects in the present setup.

After TNF α administration (3.5 hours), mice were anesthetized by subcutaneous (s.c.) administration of the ketamine/xylazine mixture (see above). Body temperature was kept at 37 °C by means of an infrared heating lamp. To enable intravital microscopic observation of leukocytes, 10-20 μ l of a Rhodamine 6G solution (1 mg/ml in 0.9% NaCl solution) was injected into the tail vein when needed. In all mice, venules (10-40 μ m) in both ears were visualized using a Leitz intravital microscope adapted for telescopic imaging¹⁵. Images were recorded on videotape for off-line analysis.

To enable quantification of systemic leukocyte counts, 20 μ l blood was sampled from the vena cava at the end of every experiment, and added to Türk's solution (Merck) in a 1:10 dilution. Leukocytes were counted and differentiated as polymorphonuclear (PMN) or monomorphonuclear (MMN) in a counting chamber (Clay Adams, Parsippany, NJ, USA).

Experimental parameters

Venular diameters were determined with a home-built image shearing device¹⁶. Centerline blood flow velocity was measured by frame-to-frame analysis, using the fastest passing fluorescent leukocyte as a marker. The level of leukocyte rolling was determined by counting the number of rolling cells passing a vessel segment per minute. Leukocytes were considered as rolling when their velocity along the vessel wall was at least an order of magnitude lower than that of the free flowing blood cells. The level of leukocyte adhesion was assessed in a 100 μ m segment of the venule, and expressed as number of cells per endothelial surface area (assuming the cross-section of the vessels to be circular). Leukocytes were considered adherent when they remained stationary for at least 30 seconds. We also examined the total number of interacting leukocytes at a particular moment; this parameter includes not only the number of adhering and rolling leukocytes, but also the velocity of the latter. This parameter was determined by counting in a frozen video frame the total number of rolling and adhering leukocytes in a 100 μ m vessel segment; at each time point, this count was performed in 4 randomly chosen video frames and the data were averaged. In the tumor bearing

ears, leukocyte-vessel wall interactions were quantified in venules in three different regions: (i) within the tumor, (ii) adjacent to the tumor (within a radius of 2.5 mm around the tumor), and (iii) at the edge of the ear. In ears without a tumor the venules were chosen at random.

Real time quantitative RT-PCR

In order to detect VEGF and bFGF gene transcription, RNA was extracted from frozen LS174T tumor tissue using a RNeasy mini kit (Qiagen, Valencia, USA) and from proliferating LS174T cells using TRIzol reagent (Invitrogen, Breda, the Netherlands). Subsequently, a real time quantitative RT-PCR was performed in order to quantitate transcription levels of VEGF and bFGF as described by Meulemans et al. (submitted).

Adhesion molecule expression on isolated endothelial cells and b.END5

To culture mouse microvascular ECs, normal (lung, heart, kidney) and tumor tissues obtained from both Swiss/nude and C57Bl/6 mice were mechanically and enzymatically digested during 1 hour at 37°C, using a mixture of 1 mg/ml collagenase and 2.5 U/ml dispase (Life Technologies). After 30 minutes of incubation, 75 µg DNase (Sigma Chemical Co.) was added for another 30 minutes. The single-cell suspensions were allowed to adhere for 3 hours to gelatin coated tissue culture flasks (Costar, Corning, USA). The remaining adherent cell population consisted of 2-15% ECs as determined by CD31 expression. Cells were cultured for three days in Dulbecco's MEM (with 1000 mg/l glucose) containing 20% fetal calf serum. In one experiment, cells obtained from normal C57Bl/6 tissues were cultured for three days in the presence of 0, 10, 25, 50 or 100 ng/ml bFGF (Sanvertech, Heerhugowaard, the Netherlands). b.END5 cells were cultured for three days with 50 ng/ml bFGF or 50 ng/ml VEGF. When applied, TNF α was added 6 hours prior to harvesting. Finally, the expression of ICAM-1 and VCAM-1 was determined flow cytometrically (see below).

FACS-analysis of endothelial and leukocyte adhesion molecules

Cells were harvested and fixed for 30 minutes in 1% paraformaldehyde (Merck) at room temperature. Afterwards, cells were resuspended in 20 µl appropriately diluted rat anti-ICAM-1 monoclonal antibody (R&D systems, Abingdon, UK) or rat anti-VCAM-1 monoclonal antibody (PharMingen, San Diego, CA) and incubated for 1 hour on ice. Subsequently, the cells were incubated for 1 hour with goat-anti-rat IgG conjugated to FITC (Pierce & Warriner, Amsterdam, the Netherlands). Finally, the cells were incubated with phycoerythrin (PE) conjugated rat anti-mouse CD31 (Dako, Glostrup, Denmark). Stained cells were analyzed on a FACS calibur flowcytometer. Data analysis was performed using Cellquest software (Becton Dickinson, Mountain View, CA).

Mouse blood leukocytes were isolated by Ficoll density gradient centrifugation (Amersham, Uppsala, Sweden). Cells were fixed for 30 minutes in 1% paraformaldehyde (Merck) at room temperature. Cells were incubated with the supernatant of the following hybridoma cell lines, R1-2 (rat anti-mouse V α -4), Mel14 (rat anti-mouse L-selectin), M17.4 (rat anti-mouse LFA-1 α chain) or M18.2 (rat anti-mouse LFA-1 β chain) (all obtained from PharMingen, San Diego, CA) for 1 hour at 4°C. Subsequently, the cells were incubated with FITC conjugated goat anti-rat IgG (Pierce & Warriner, Amsterdam, the Netherlands) for 1 hour at 4°C.

Statistics

Because of their non-symmetrical distribution, data obtained by intravital microscopic experiments are presented as medians with interquartile ranges (i.e. the spread from the 25th to 75th percentile). Data obtained by FACS analysis are presented as means with corresponding standard error of the

mean (SEM). Differences between two independent data groups were tested with the Mann-Whitney U test. Differences between paired data groups were tested with the Friedman test (more than two groups), followed by a multiple-comparison procedure. Correlation between variables was determined using Spearman's correlation test. In all tests the level of significance was set at 0.05.

RESULTS

Leukocyte-vessel wall interactions are inhibited in tumor vessels

Previously, we showed that angiogenic factors downregulate leukocyte-vessel wall interactions *in vivo*¹⁴. To investigate whether this observation also holds in the presence of a growing tumor, we developed a non-invasive mouse tumor model that can be used for intravital microscopic determination of leukocyte interactions with the microvascular endothelium.

LS174T human colon carcinoma tumor cells were injected in the ears of nude mice (Figure 2.1A and B), and after the outgrowth of small tumors TNF α -induced leukocyte-vessel wall interactions as well as local fluid dynamic parameters were examined in ear skin vessels. In the tumor-free control group, the median level of leukocytes interacting with the vascular endothelium after treatment with TNF α was 813 leukocytes/mm². Inside the ear tumor, this level was significantly lower (382 leukocytes/mm², $p < 0.05$). The total level of leukocyte-vessel wall interactions increased as a function of distance from the tumor (significant correlation of 0.35 (Rs); $p < 0.01$), being 728 leukocytes/mm² in areas adjacent to the tumor and 953 leukocytes/mm² at the outer edge of the ear. The latter level is comparable to the one measured in the contralateral ears (885 leukocytes/mm²) and with the level measured in the control group (Figure 2.1C). Examples of intravital microscopic images of control and tumor vessels are presented in Figures 2.1D and 1E. Without TNF α pretreatment, the total level of interacting leukocytes in ear skin venules was 318 cells/mm² (not shown), which indicates that the tumor microenvironment completely eradicated the TNF α -induced inflammatory response. These results demonstrate a local effect of a small tumor on leukocyte interactions with the vascular endothelium.

The total level of leukocyte-vessel wall interactions includes both leukocyte rolling and adhesion. Discrimination between these two processes revealed that the reduction in leukocyte-vessel wall interactions inside a tumor is due to an effect on leukocyte adhesion (Figure 2.2A). Whereas the level of leukocyte adhesion in tumor vessels was drastically diminished to almost undetectable levels as compared to venules in the contralateral ear (375 leukocytes/mm²) and in the control group (331 leukocytes/mm²), such differences were not found at the level of leukocyte rolling (Figure 2.2B).

We showed previously that angiogenic factors are able to downregulate leukocyte-vessel wall interactions¹⁴. Using TaqMan real time quantitative RT-PCR we were able to show that LS174T tumors, grown *in vivo* as well as cultured *in vitro*, show mRNA expression of VEGF. mRNA expression of bFGF was below detection level. To investigate whether VEGF contributed to the effect of a tumor on leukocyte-vessel wall interactions, mice bearing a small LS174T ear tumor were treated with an anti-VEGF antibody. In these mice the median level of leukocyte-vessel wall interactions was 796 cells/mm² in the tumor vessels, which is significantly higher ($p < 0.05$) compared to the non-treated ear tumor group. In the vessels adjacent to the tumor (1592 leukocytes/mm²), at the edge of the ear (1375 leukocytes/mm²) of the contralateral ear (1157 leukocytes/mm²) the level of interaction was not influenced by anti-VEGF treatment.

To investigate whether leukocyte-vessel wall interactions are similarly influenced in mice with an intact immune system, similar experiments were performed in the B16F10 mouse melanoma

Figure 2.1: Leukocyte-vessel wall interactions are diminished in tumors. (A and B) Small tumors were grown in ears of Swiss/nude mice (as indicated by the arrows). (C) Interacting leukocytes were quantified in different areas of tumor bearing and control mice (all treated with 750 ng TNF α). Data are presented as medians and interquartile ranges.

* $p < 0.05$ as compared to the control group. # $p < 0.05$ as compared to the edge of the ear. Images of intravital fluorescence microscopy are shown of a venule in a contralateral ear (D) and a vessel in a small ear tumor (E); note the large difference in leukocyte-vessel wall interactions between control and tumor vessels. Leukocytes were fluorescently labeled using Rhodamin 6G. The bars represent 25 μ m. Video images can be observed at the website of our laboratory: <http://www.fdg.unimaas.nl/angiogenesislab>. For numbers of mice and vessels see Table 2.1.

model in C57BL/6 mice. These experiments revealed comparable results, showing reductions in total level of interacting and adhering leukocytes in tumor vessels as compared to vessels outside the tumor (significant correlation of 0.55 (Rs) between leukocyte-vessel wall interactions and distance to the tumor; $p < 0.01$) and in tumor-free control mice (Figure 2.3A and B). Interestingly, in the immunocompetent mice we did find a significant inhibition in the level of rolling leukocytes inside the tumor (3 leukocytes/min) as compared to the edge of the ear (15 leukocytes/min, $p < 0.05$) (Figure 2.3C).

Figure 2.2: Leukocyte adherence but not rolling is diminished in ear tumor vessels in TNF α treated Swiss/nude mice (750 ng TNF α /mouse). (A) Number of adhering leukocytes per mm² and (B) number of rolling leukocytes per minute in ear venules. Data are presented as medians and interquartile ranges. For numbers of mice and vessels see Table 2.1.

* $p < 0.05$ as compared to the control group. # $p < 0.05$ as compared to the edge of the ear.

A large tumor has a systemic effect on leukocyte-vessel wall interactions

Injection of LS174T tumor cells on the flank of a nude mouse resulted in a large (about 1500 mm³), non-infiltrating and non-metastasizing tumor within approximately three weeks. Analysis of the ear venules of this group lead to the surprising finding of a significantly suppressed level of leukocyte-vessel wall interactions (509 leukocytes/mm², $p < 0.05$), as compared to the control group without a tumor (813 leukocytes/mm², $p < 0.05$) (Figure 2.4A). Again, comparable to the situation in the small ear tumors, this reduced level of leukocyte-vessel wall interactions can be attributed to a diminished level of leukocyte adhesion (Figure 2.4B). The level of leukocyte rolling was not found different from control (Figure 2.4C).

These experiments were also performed in the B16F10 mouse melanoma model in C57Bl/6 mice. A significant reduction of leukocyte-vessel wall interactions was found in mice with a large flank tumor (1364 leukocytes/mm²) as compared to the control mice without a tumor (1842 leukocytes/mm², $p < 0.05$) (Figure 5A). This decline of leukocyte-vessel wall interactions can also be attributed to a decreased level of leukocyte adhesion as compared to the control group (Figure 2.5B). No significant differences were found in leukocyte rolling (Figure 2.5C).

Leukocyte adhesion molecule expression, systemic leukocyte counts and fluid dynamic parameters

One explanation for the observed tumor effect on leukocyte-vessel wall interactions could be that leukocytes themselves are affected by the presence of a tumor. To exclude this effect, we investigated adhesion molecule expression on circulating leukocytes in mice bearing a small ear tumor and in healthy controls. We found no difference in expression of LFA-1 α , LFA-1 β , VLA-4 or L-selectin between these two groups (data not shown), suggesting that the decreased level of interacting leukocytes we observe inside an ear tumor is not due to altered adhesion molecule expression on leukocytes. TNF α induced a decrease in the number of circulating leukocytes from 2.4×10^6 leukocytes/ml to 0.9×10^6 leukocytes/ml ($p = 0.05$) in nude mice. This is probably due to the fact that TNF α induces

leukocyte adhesion systemically. This effect was evident for both MMNs and PMNs, but was most prominent for MMNs (80% reduction versus 55% for PMNs ($p < 0.01$)). In contrast to a small ear tumor (1.3×10^6 leukocytes/ml), the presence of a large flank tumor tended to mask these TNF α induced changes (1.8×10^6 leukocytes /ml), also suggesting a systemic effect of the tumor.

No significant differences in blood flow velocities were detected between the vessels of the various experimental groups of both Swiss/nude and C57Bl/6 mice (Table 2.1), except for deviating centerline velocities in the vessels inside the tumor in both mouse models. However, no correlation between centerline velocity values and any of the leukocyte-vessel wall interaction related parameters could be found in any of the groups. Therefore, the observed differences in leukocyte-vessel wall interactions cannot be explained by differences in fluid dynamic parameters.

Diminished leukocyte adhesion in tumors is the result of suppressed endothelial adhesion molecule expression

In previous studies, it was demonstrated that human tumor ECs express suppressed levels of adhesion molecules that are involved in leukocyte-vessel wall interactions^{4,11}. In the present study we investigated the expression of ICAM-1 (an important adhesion molecule involved in leukocyte emigration) on tumor ECs. To this purpose, ECs were freshly isolated from LS174T and B16F10 tumors and from normal tissues. Adhesion molecule expression on ECs was determined by double staining, using CD31 and ICAM-1 antibodies, and flow cytometry⁴. ICAM-1 expression was found to be downregulated in tumor associated endothelium as compared to ECs obtained from normal tissue (Figure 2.6A and B).

Figure 2.3: Leukocyte adherence and rolling are both diminished in tumor vessels in TNF α treated C57Bl/6 mice (500 ng TNF α /mouse). (A) Total level of interacting leukocytes per mm², (B) number of adhering leukocytes per mm² and (C) number of rolling leukocytes per minute in ear venules. Data are presented as medians and interquartile ranges. For numbers of mice and vessels see Table 2.1.

* $p < 0.05$ and *** $p < 0.001$ as compared to the control group.

Figure 2.4: A large flank tumor has a systemic effect on leukocyte-vessel wall interactions in $TNF\alpha$ treated Swiss/nude mice (750 ng $TNF\alpha$ /mouse). (A) Level of interacting leukocytes per mm^2 , (B) number of adhering leukocytes per mm^2 and (C) number of rolling leukocytes per minute in ear venules. Data are presented as medians and interquartile ranges. For numbers of mice and vessels see Table 2.1. * $p < 0.05$ as compared to the control group.

Figure 2.5: A large flank tumor has a systemic effect on leukocyte-vessel wall interactions in $TNF\alpha$ treated C57Bl/6 mice (500 ng $TNF\alpha$ /mouse). (A) Level of interacting leukocytes per mm^2 , (B) number of adhering leukocytes per mm^2 and (C) number of rolling leukocytes per minute in ear venules. Data are presented as medians and interquartile ranges. For numbers of mice and vessels see Table 2.1. * $p < 0.05$ as compared to the control group.

Whereas ICAM-1 levels in the tumor were low (mean fluorescence intensity of 2 in LS174T tumors and 1.5 in B16F10 tumors), in normal tissues these values ranged between 40 and 57. In order to investigate whether angiogenic factors produced by the tumor are responsible for this downregulation of ICAM-1, normal tissue ECs were cultured for three days in the presence of various concentrations of bFGF (Figure 2.6C). bFGF was observed to downregulate ICAM-1 on freshly isolated ECs in a dose dependent manner. In separate experiments, the mouse EC line b.END5 was cultured with or without bFGF or VEGF and in presence or absence of TNF α (Figure 2.6D). In the absence of TNF α , bFGF was observed to downregulate ICAM-1 expression ($p < 0.05$). On the contrary, TNF α was able to significantly upregulate both ICAM-1 ($p < 0.01$) and VCAM-1 ($p < 0.01$). The TNF α -induced upregulation of ICAM-1 and VCAM-1 was reduced in cells that were pre-incubated with bFGF (ICAM-1: $p < 0.05$, VCAM-1: $p = 0.07$) or VEGF (ICAM-1: $p = 0.06$, VCAM-1: $p = 0.06$). These results suggest angiogenic factors as regulators of leukocyte-vessel wall interactions.

DISCUSSION

The present study demonstrates, in two separate mouse models, that leukocyte-vessel wall interactions are locally suppressed within a tumor. Surprisingly, large tumor burdens were observed to generate a systemic downregulation of leukocyte-vessel wall interactions. The reduction in leukocyte-vessel wall interactions in tumor vessels appeared to be caused by a decreased expression of adhesion molecules on endothelial cells (ECs), and not by suppression of adhesion molecules on leukocytes. Our finding that pre-stimulation with bFGF or VEGF reduces the TNF α -induced expression of ICAM-1 and VCAM-1 on cultured mouse ECs strongly suggests that tumor derived growth factors are involved. A reduced level of leukocyte-vessel wall interactions is likely to result in impaired extravasation and infiltration of leukocytes into the tumor. Through this mechanism a tumor has the opportunity to escape the immune system.

We developed a new tumor model in which leukocyte-vessel wall interactions in and outside a tumor can be measured non-invasively, allowing longitudinal analysis during tumor development and/or in the course of an anti-cancer treatment. Several other investigators have also used animal models to examine leukocyte-vessel wall interactions in the presence of a tumor¹⁷⁻²³. However, these studies used invasive techniques such as dorsal skin fold chambers, cranial windows or skin-flaps. These techniques are more time-consuming, carry a higher risk of failure and may induce an inflammatory reaction by surgical procedures at the site of interest. In the present study, athymic nude mice were selected to enable implantation of a tumor of human origin. These mice have large transparent hairless ears, which is a clear advantage for intravital microscopic measurements. The use of athymic mice to study immune functions may be problematic, because of limited cognate immunity due to the lack of mature T-lymphocytes. However, innate immunity (i.e. granulocytes, monocytes and natural killer cells) and B-lymphocytes are present and functional in this model. Nevertheless, we repeated the experiments using syngeneic B16F10 melanoma cells in immune competent C57Bl/6 mice. This model was chosen because of the availability of a large body of literature on immunological anti-tumor responses in these mice²³⁻²⁶.

It has previously been demonstrated that the expression of adhesion molecules is suppressed in human tumor ECs^{6,27,28}. Furthermore, we have shown earlier *in vitro*⁴ and *in vivo*¹⁴ that this is most probably due to differences in metabolic state and phenotype of tumor EC by exposure to tumor-derived angiogenic factors such as basic fibroblast growth factor (bFGF) and vascular endothelial cell growth factor (VEGF). Other studies have shown that bFGF reduces monocyte and natural killer cell adhesion and migration by downregulating adhesion molecules on human

Table 2.1: Fluid dynamic parameters in venules in both ears of TNF α -treated Swiss/nude mice and C57Bl/6 mice.

		Ear tumor		Flank tumor		Control	
		Within tumor	Adjacent tumor	Edge ear	Contra-lat. ear		
Swiss/nude	n_m	6	6	6	6	4	4
	n_v	17	20	21	31	60	48
	Diameter (μ m)	20 (20-26)	24 (20-29)	25 (20-27)	25 (22-29)	23 (20-27)	25 (23-28)
	Centerl. vel. (mm/s)	1.0 (0.6-1.3)*	0.6 (0.5-1.0)	0.6 (0.5-0.8)	0.6 (0.5-1.1)	0.6 (0.4-0.8)	0.6 (0.4-1.3)
C57Bl/6	n_m	7	7	7	7	4	4
	n_v	19	24	21	15	46	41
	Diameter (μ m)	15 (10-17)	20 (19-35)	22 (19-28)	24 (17-25)	24 (20-27)	25 (20-30)
	Centerl. vel. (mm/s)	0.3 (0.2-0.6)*	0.4 (0.2-0.6)	0.6 (0.4-0.8)	0.5 (0.4-0.6)	0.6 (0.4-0.8)	0.6 (0.5-1.3)

Data are presented as median values and interquartile ranges in parentheses.

n_m =number of mice, n_v = number of vessels.

* $p < 0.05$ as compared to the control group.

umbilical vein endothelial cells (HUVECs)^{7,29}. Here, we show that in our mouse models tumor ECs also have suppressed ICAM-1 expression. In addition, TNF α -induced upregulation of ICAM-1 and VCAM-1 was reduced in cultured b.END5 cells (mouse endothelioma cells) pretreated with either bFGF or VEGF. Since we also show that LS174T tumor cells contain mRNA for VEGF, while it is known from literature that B16F10 tumor cells express high levels of bFGF and VEGF^{30,31}, our data strongly suggest that the downregulation of leukocyte-vessel wall interactions in tumor vessels, as observed in the present study, is a result of exposure of ECs to these angiogenic factors. This hypothesis is supported by our observation that treatment of mice bearing a small ear tumor with humanized anti-VEGF antibody prevents the downregulation of leukocyte-vessel wall interactions inside the tumor.

Our finding that leukocyte-vessel wall interactions are decreased inside a tumor was confirmed in several previous studies¹⁷⁻²². In literature, however, there is some dispute with regard to the effects of VEGF on the expression of adhesion molecules on ECs. Whereas the results of some studies are in agreement with our data and show a role for VEGF as an inhibitor of leukocyte-vessel wall interactions³², the results of other studies seem to be in contrast with our findings^{7,33}. These latter studies report an increase in adhesion molecule expression on HUVEC after VEGF stimulation. However, in these studies VEGF stimulation was always 24 hours or less. In a pilot study, we also observed an initial upregulation of ICAM-1 expression after VEGF stimulation of HUVECs; in contrast, prolonged stimulation (which is also the case in the presence of a tumor) results in a downregulation of ICAM-1 expression and leukocyte adhesion (data not shown). In one other study, performed by Detmar and colleagues³⁴, leukocyte-vessel wall interactions were found to be increased in skin vessels of VEGF-transgenic mice. However, this experimental approach in which the effects of a continuous overexpression of VEGF were investigated is completely different from the situation within tumors where VEGF (and also other (growth) factors) is produced locally in increasing quantities.

The present study is the first to show a systemic effect of a large tumor: leukocyte-vessel wall interactions appeared to be downregulated in ear microvessels of mice bearing a large

tumor on their flank. This systemic effect is less pronounced as compared to the situation within a tumor; leukocyte adhesion was only partially downregulated, while inside a tumor the level of adhesion was reduced to zero. This may be due to the fact that the circulating concentration of angiogenic factors in mice with a large flank tumor is lower than the local

Figure 2.6: ICAM-1 is downregulated on mouse ECs isolated from a tumor as compared to ECs isolated from normal tissue. Normal and tumor tissues from Swiss/nude (A) and C57Bl/6 mice (B) were mechanically and enzymatically digested. The obtained single-cell suspensions were cultured for three days. Subsequently, the cells were harvested and the expression of ICAM-1 on ECs was determined by FACS analysis. Data were divided by control values and presented as means of fluorescence intensity and corresponding standard error of the mean (SEM) values. (C) Normal C57Bl/6 ECs were cultured with various concentrations of bFGF. Subsequently, the cells were harvested and the expression of ICAM-1 on ECs was determined by FACS analysis. (D) b.END5 cells were cultured for three days with 50 ng/ml bFGF or 50 ng/ml VEGF. TNF α was added 6 hours prior to harvesting. Afterwards, the cells were harvested and the expression of ICAM-1 and VCAM-1 on ECs was determined by FACS analysis. * $p < 0.05$, ** $p < 0.01$ as compared to the control group, # $p < 0.05$, ^ $p \leq 0.07$ as compared to TNF α group.

concentration inside a small ear tumor. The fact that the systemic leukocyte count was higher in TNF α -treated mice with a large tumor on their flank than in TNF α -treated control mice also suggests a systemic inhibitory effect of the flank tumor on leukocyte-vessel wall interactions. Since it is known for that some tumors produce soluble TNF α -receptors^{35,36}, this might also be an explanation for the observed systemic effect of a large tumor on leukocyte-vessel wall interactions. However, we also show data (Figure 2.6D) on the direct effect of angiogenic factors on EC, which exclude tumor cell interference in this process. Therefore we believe that the observed effects are explained by an endothelial cell mediated mechanism rather than a mechanism controlled by tumor derived signals.

The current results indicate that in the nude mouse model adhesion but not rolling is modulated by tumor derived factors. This suggests that ICAM-1 but not the selectins are sensitive to regulation by these factors, which would be a discrepancy with the human situation, where E- and P-selectin were found to be suppressed in tumor ECs^{11,37}. It is more likely that the absence of effects on leukocyte rolling is due to selection of the nude mouse model, because we did observe a significant effect on leukocyte rolling inside the tumor in C57Bl/6 mice. We hypothesize that either tumor regulation of leukocyte rolling is confined to a functional T cell population or that this effect is dependent on specific (combinations of) cytokines that are expressed by an intact immune system. However, the observed differences between the two tumor models on leukocyte rolling may also be due to other mouse strain-specific characteristics.

During its development a growing tumor needs to undergo the so-called angiogenic switch, which is the starting point of the attraction of new blood vessels to grow beyond the size of 1-2 mm in diameter. It is possible that the small ear tumors studied in our experiments did not display a full angiogenic profile. Although this certainly would be an issue for spontaneous tumor models, in our model the injected cells already expressed VEGF and/or bFGF during culture. Evidence for the fact that we injected the cells in an advanced angiogenic state is provided by the fact that we observed the formation of an haematoma-like spot within 24 hours after injection, which might be due to a permeability increase induced by VEGF, and the presence of new vasculature already after 2-3 days (Figure 2.1A and B). Moreover, it was established that the small ear tumors are able to grow to the size of more advanced tumors (diameter >3 mm), indicating that angiogenic factors are being excreted by the tumor cells. Also, the results we obtained using real time quantitative RT-PCR show that LS174T cells contain mRNA for VEGF in culture already. Therefore, we favor the view that our ear tumor model is relevant for the study of the early outgrowth of metastases rather than the outgrowth of a primary tumor.

In conclusion, the currently described tumor models introduce a non-invasive method for studying leukocyte-vessel wall interactions *in vivo*. We have demonstrated that leukocyte-vessel wall interactions are suppressed within a tumor, but increase as a function of distance from the tumor. Moreover, in case of a large tumor burden there appears to be a systemic downregulation of leukocyte interactions with the vessel wall. This effect is mediated by downregulation of endothelial adhesion molecules, probably by angiogenic factors, leading to the escape of tumors from the immune system. Future studies will address the issue of the reversal of the observed phenomena by treatment with angiogenesis inhibitors and the improvement of (adoptive) immunotherapy by inhibitors of angiogenesis as a novel treatment strategy for cancer.

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CHAPTER 3

Anti-angiogenesis therapy can overcome endothelial cell anergy and promote leukocyte-endothelium interactions and infiltration in tumors

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ABSTRACT

Tumor escape from immunity, as well as the failure of several anti-cancer vaccination and cellular immunotherapy approaches, is suggested to be due to the angiogenesis mediated suppression of endothelial cell (EC) adhesion molecules involved in leukocyte-vessel wall interactions. We hypothesized that inhibition of angiogenesis would overcome this escape from immunity. We investigated this *in vivo* by means of intravital microscopy and *ex vivo* by immunohistochemistry in two mouse tumor models. The angiogenesis inhibitors anginex, endostatin and angiostatin, and also the chemotherapeutic agent paclitaxel were found to significantly stimulate leukocyte-vessel wall interactions by circumvention of EC anergy *in vivo*, i.e. by the upregulation of endothelial adhesion molecules in tumor vessels. This was confirmed by *in vitro* studies on cultured EC at the protein and mRNA levels. The new angiostatic designer peptide anginex was most potent at overcoming EC anergy; the enhanced leukocyte-vessel interactions led to an increase in the numbers of tumor infiltrating leukocytes. While anginex inhibited tumor growth and microvessel density (MVD) significantly, the amount of infiltrated leukocytes (CD45), as well as the number of CD8+ cytotoxic T-lymphocytes, was enhanced markedly. The current results suggest that immunotherapy strategies can be improved by combination with anti-angiogenesis.

INTRODUCTION

Over the last decades, immuno-directed anti-tumor strategies, based on adoptive- or vaccination approaches, have been developed ^{1,2}. This approach has not been as effective as had been anticipated. Several explanations for this have been put forward. Firstly, most vaccines have been directed towards stimulating cytotoxic T-lymphocyte (CD8) responses and the continuous stimulation of these cells without T-cell help (CD4) eventually leads to anergy and tumor escape. Secondly, regulatory host T-cell responses may counteract induced immunity. Thirdly, the antigens towards which the immunity is directed are not tumor-specific enough. An alternative explanation might be that although immune effector cells are being generated *in vivo*, they are unable to migrate into tumor sites and therefore cannot exert an anti-tumor effect.

Angiogenesis, a pivotal process in the outgrowth and metastasis of tumors ^{3,5}, has been described to be involved in the escape of tumors from immune surveillance. We and others have previously shown that tumor EC have a suppressed expression of adhesion molecules (EC anergy ⁶), such as intercellular adhesion molecule-1/-2 (ICAM-1/-2), vascular endothelial cell adhesion molecule-1 (VCAM-1) and CD34 ^{7,9}, due to exposure to angiogenic factors such as vascular EC growth factors (VEGFs) and fibroblast growth factors (FGFs) ^{6,10,11}. We have recently demonstrated in several mouse tumor models that tumors indeed inhibit leukocyte-vessel wall interactions by downregulation of vascular adhesion molecules ¹².

The role of angiogenesis in anti-tumor immunity is an emerging theme in both angiogenesis and immunology research fields. This is exemplified by recent studies on the major prognostic value of T-lymphocyte infiltration in tumors and the relationship with expression of angiogenic factors ¹³, immunization against angiogenesis related proteins ^{14,15} and ECs as vaccines ¹⁶. In this study, we use several angiostatic molecules to demonstrate that angiogenesis inhibition can be a way to increase leukocyte-vessel wall interactions and subsequent infiltration. Angiex is a designed 33-mer peptide angiogenesis inhibitor that inhibits vasculature formation by induction of apoptosis in endothelial cells ^{17,18}. Angiostatin and endostatin are two other angiogenesis inhibitors with a direct effect on endothelial cells ¹⁹⁻²¹. TNP-470 was described in the early 90s as an angiostatic derivative of the fungus antibiotic fumagillin ^{22,23}. Also several chemotherapeutic agents with claimed angiostatic activity ²⁴ such as paclitaxel and cyclophosphamide were included in these studies. It is suggested that the stimulating effect of angiogenesis inhibition on leukocyte-vessel wall interactions may be applicable for the improvement of various immunotherapy approaches.

METHODS

Cell culture

Human LS174T colon carcinoma cells were grown in DMEM (Life Technologies, Paisley, Scotland), supplemented with 10 % fetal calf serum (FCS, Bio Whittaker, Verviers, Belgium), 1% glutamin. Mouse B16F10 melanoma cells (kindly provided by dr. J. Fidler, Houston, Texas) were cultured using Hank's MEM (Life Technologies, Paisley, Scotland) containing 5% FCS, 1% non-essential amino acids (Life Technologies), 1% sodium pyruvate (Life Technologies), 1.5 % MEM vitamins (Life Technologies), and 2% sodium bicarbonate (Life Technologies).

Mouse b.END5 brain endothelioma cells (obtained from ECACC, Salisbury, United Kingdom) were cultured in Dulbecco's MEM (Life Technologies) containing 10% FCS, 1% glutamine (Life Technologies, Paisley, Scotland), 5 μ mol/l 2-mercaptoethanol (Sigma, St Louis, MO), 1% non-essential amino acids (Life Technologies) and 1 mmol/l sodium pyruvate (Life Technologies). All cells were

harvested using 0.25% trypsin (Difco Laboratories, Detroit, MI)/0.02% EDTA (Merck, Darmstadt, Germany) in 0.9% NaCl solution.

Human umbilical vein endothelial cells (HUVEC) were cultured in RPMI-1640 (Life Technologies, Breda, The Netherlands) supplemented with 20% heat inactivated human pooled serum (provided by the University Hospital Maastricht), 2 mM L-glutamin (Life Technologies), 50 ng/ml streptomycin and 50 U/ml penicillin (ICN Biomedicals, Maidenhead, UK) in 0.2% gelatin coated tissue culture flasks at 37°C, 5% CO₂.

Intravital microscopy

In vivo experiments were approved by the local Ethical Review Committee on Animal Experiments. Small tumors were grown in the left ear of C57BL/6 mice (25-30 g, obtained from Charles River, Maastricht, the Netherlands), as described before ¹². Briefly, 10 µl cell suspension containing 10⁶ B16F10 cells was injected intradermally into the dorsal side of the ear. Within 3-5 days ear tumors grew to a diameter of about 2 mm, at which time point the treatment started. Three anti-angiogenic drugs were used, i.e., the designer peptide anginex ^{17,25}, endostatin and angiostatin (EntreMed, Rockville, MD) ²⁶, and TNP-470 (Takeda Chemical Industries, Osaka, Japan) ²⁷, as well as two anti-neoplastic drugs with claimed anti-angiogenic properties, i.e. paclitaxel (Taxol, Bristol-Myers Squibb B.V., Woerden, the Netherlands) and cyclophosphamide (Asta Medica, Diemen, the Netherlands).

For treatment of tumor bearing mice with anginex (n=6), Alzet osmotic minipumps (Durect Corporation, Cupertino, USA) were implanted on the flank on day 5 after tumor cell administration. Pumps administered 6 mg/kg/day of anginex, a dose that proved to inhibit tumor growth in this model by approximately 70-80% ¹⁷. Other groups of mice were similarly treated with endostatin (2 mg/kg/day, n=5) and angiostatin (2 mg/kg/day, n=5) ^{26,28}. In two additional mice an osmotic minipump was implanted that administered equivalent amounts of control protein (bovine serum albumin). In these groups, intravital microscopic measurements were performed on day 12 after tumor cell inoculation. TNP-470 was administered according to a previously optimized protocol ²⁷, by i.p. injections of 20 mg/kg (in 5% glucose/ethanol solution) on days 5, 8, 11 and 13 after tumor cell administration (n=4). In these mice intravital microscopy was performed on day 14. Paclitaxel (6 mg/kg in 0.9% NaCl solution, i.p.; n=7) and cyclophosphamide (12.5 mg/kg in sterile water, i.p.; n=4) were administered on days 5 and 7 after tumor cell administration. Intravital microscopy was performed on day 8, at which time point tumor growth was already inhibited significantly [Bouma-ter Steege, unpublished results], while no toxic side effects were observed. As a control group, 5 tumor bearing mice were used which were not treated with any of the anti-angiogenic or anti-neoplastic compounds, but treated similarly otherwise. To investigate the effect of the drugs in healthy blood vessels, we also used mice without a tumor and treated them with anginex (n=2), endostatin (n=2), angiostatin (n=2), TNP-470 (n=2), paclitaxel (n=4) and cyclophosphamide (n=2); as control 5 mice were left untreated.

On the day of the experiment, mice received 500 ng recombinant murine TNFα (R&D Systems, Abingdon, U.K.) i.p., 4 hours prior to intravital microscopy. Administration of TNFα was performed to upregulate leukocyte-vessel wall interactions; without cytokine pretreatment adhering leukocytes are hardly present in mouse ear venules, which would preclude the assessment of a possible reduction in leukocyte adhesion ²⁹. Mice were anesthetized by s.c. administration of a mixture of ketamine (0.1 mg/g b.w. Nimatek) and xylazine (0.02 mg/g b.w. Sedamun, Ad Usem Veterinarium, Cuyk, the Netherlands). Intravital microscopy was performed as described before ¹². Briefly, body temperature was kept at 37°C by an infrared heating lamp. In all mice, venules (10-40 µm) in both ears were visualized using a Leitz intravital microscope adapted for telescopic imaging ³⁰. To enable

intravital microscopic observation of leukocytes, 10-20 μ l of a Rhodamine 6G solution (Sigma Chemical Co., St. Louis, MO; 1 mg/ml in sterile 0.9% NaCl) was injected into a tail vein. As many vessels as possible (typically 8-12) were recorded for 3-5 minutes each, in areas within the tumor, outside the tumor (<2.5 mm from the tumor), at the edge of the ear (>5 mm from the tumor) and in the contralateral tumor-free ear. To enable quantification of systemic leukocyte counts, 20 μ l blood was sampled via heart puncture at the end of every experiment. Leukocytes were counted and differentiated as polymorphonuclear (PMN) or monomorphonuclear (MMN).

Experimental parameters

Vessel diameters were measured using an in-house built image shearing device. Centerline blood flow velocity was determined by frame-to-frame analysis, using the fastest passing fluorescent leukocyte as a marker. The level of leukocyte rolling was determined by counting the number of rolling cells passing a vessel segment per minute. Leukocytes were considered as rolling along the vessel wall when their velocity was at least an order of magnitude lower than that of the free flowing blood cells. The level of leukocyte adhesion was assessed in a 100 μ m vessel segment, and expressed as number of cells per endothelial surface area (assuming the cross-section of the venules to be circular). Leukocytes were considered adherent when they remained stationary for at least 30 seconds. The total number of leukocytes interacting with the vessel wall at a particular moment was determined as well. This parameter includes not only the numbers of adhering and rolling leukocytes, but also the velocity with which the latter roll along the endothelium within a vessel segment of 100 μ m length. When leukocytes roll slowly, the number of interacting leukocytes observed in such a vessel segment will be higher than when they roll faster, despite similar numbers of rolling and adhering leukocytes. This information is important, because it tells something about the adhesion molecules involved; leukocytes are known to roll very slowly on E-selectin, whereas rolling on P-selectin is much faster. In addition, slowly rolling leukocytes are the first to become adherent. To determine this parameter, we counted in a frozen video frame the total number of rolling and adhering leukocytes in a 100 μ m vessel segment. At each time point, this count was performed in 4 randomly chosen video frames and the data were averaged.

Tumor growth experiments

At day 0, 10^6 LS174T human colon carcinoma cells or 2×10^5 B16F10 melanoma cells were inoculated subcutaneously on the right flank of 6 week old Swiss nu/nu or C57BL/6 mice, respectively. At day 5, when established tumors were present (approx. 50 mm³), an Alzet osmotic minipump (Durect, Cupertino, CA) was placed on the left flank. For LS174T tumors, treatment was performed for 2 weeks with 6 mg/kg/day anginex in 0.9% NaCl solution (saline). In the B16F10 tumors, treatment was performed for 11 days with a dose range of 0, 2, 6, or 12 mg/kg/day of anginex in saline). Tumor volumes were measured daily and calculated as follows: width² X length X 0.52. At the end of experiments tumors were excised and snap frozen for histochemical analysis. Statistical differences in tumor growth curves were analyzed using the two way ANOVA test.

Immunohistochemistry

Five μ m sections were made, dried overnight, and fixed in acetone for 15 minutes at -20°C. After blocking of endogenous peroxidase, sections were blocked with BSA for aspecific staining (in 1%BSA/20%FCS/0.1%Tween-20 in PBS for 30 minutes) and stained using rat anti-mouse CD31 (PharMingen, San Diego, CA), CD8 or CD45 (both kind gifts from Dr. A Duijvestijn, Maastricht). CD31 was detected by HRP-labelled goat anti-rat Ig antibody (Chemicon), whereas CD8 and CD45

were detected by biotinylated donkey anti-rat Ig antibody (Jackson Immuno Research Laboratories, Inc) and avidin-biotin-HRP complex (Dako). Microvessel density (MVD), CD45⁺ and CD8⁺ cells were counted in 4 independent areas in each section (using a 0.25 mm² grid at a 200 times magnification) by two independent observers. Statistical analysis was done using the Mann-Whitney U test.

FACS analysis

b.END5 cells were cultured with or without 50 ng/ml bFGF, with or without either 30 or 10 µg/ml anginex, 500 µg/ml endostatin, 1 mg/ml angiostatin, 100 ng/ml TNP-470, or 10 ng/ml paclitaxel. 40 ng/ml TNFα was added 6 hours prior to harvesting. ICAM-1 and VCAM-1 expression was determined by rat anti-mouse ICAM-1 (CD54; R&D systems) or rat anti-mouse VCAM-1 (CD106; Pharmingen) monoclonal antibodies, as described previously¹². Similar experiments were done with HUVECs, using 10 ng/ml bFGF. ICAM-1 was detected by MEM111 mouse anti-human ICAM-1 antibody (Monosan, Uden, The Netherlands). Mouse blood leukocytes were isolated by Ficoll density gradient centrifugation (Amersham, Sweden), and incubated with R1-2 rat anti-mouse VLA-4, Mel-14 rat anti-mouse L-selectin, M17.4 rat anti-mouse LFA1α, or M18.2 rat anti-mouse LFA1, (Pharmingen), as described before¹².

Quantitative real time RT-PCR (qRT-PCR)

qRT-PCR was performed with murine specific primers³¹ detecting beta-actin, cyclophilin, ICAM-1, VCAM-1, and E-selectin (Sigma-Genosys, UK) and analyzed with Sequence Detection System software (Applied Biosystems). The expression of each target gene was normalized to the control genes.

Statistics

Data obtained from intravital microscopic experiments are presented as medians with interquartile ranges (i.e. the spread from the 25th to 75th percentile). Other data are presented as means with standard errors (SEM). Differences between two independent data groups were tested with the Mann-Whitney U test. Differences between paired data groups were tested with the Friedman test (more than two groups), followed by a multiple-comparison procedure. Correlation between variables was determined using Spearman's correlation test. In all tests the level of significance was set at 0.05.

RESULTS

Leukocyte-vessel wall interactions are reduced in tumor vessels in vivo; restoration by anti-angiogenesis treatment

It was previously shown in different mouse models that pro-angiogenic factors reduce cytokine (TNFα) induced leukocyte-vessel wall interactions in tumors and also in control tissues^{10,12}. To investigate whether angiogenesis inhibitors can revert this process in tumors, tumor bearing mice were treated with the angiostatic peptide anginex, using a protocol (6 mg/kg/day for 7 days) which inhibits tumor growth by 70-80%¹⁷. Interestingly, this treatment completely restored the total level of cytokine induced leukocyte-vessel wall interactions inside the tumor ($p < 0.001$), as well as in vessels adjacent to the tumor ($p < 0.05$), to the level observed in healthy control mice (Figure 3.1). Discrimination between leukocyte rolling and adhesion revealed that treatment with anginex significantly increased both leukocyte adhesion ($p < 0.05$) and rolling ($p < 0.01$, Figure 3.1) in tumor vessels. Interactions in the contralateral ears of tumor bearing mice were highly similar to results observed in healthy control mice (data not shown). In ear skin venules of mice without a tumor anginex did not significantly

Figure 3.1: Leukocyte-vessel wall interactions are suppressed in tumor microvessels; normalization by treatment with anginex.

C57Bl6 mice with established B16F10 tumors were treated for 7 days with anginex, bovine serum albumin (BSA) as protein control or received no treatment at all. Leukocyte vessel wall interactions were analyzed using intravital microscopy, 4 hours after i.v. injection with 500 ng TNF α . Effects of treatment on the total level of interacting leukocytes (A), on leukocyte adhesion (B) and on leukocyte rolling (C). Data are presented as medians and interquartile ranges. Statistical significance was assessed in comparison to values in untreated control mice (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

influence the total level of leukocyte-vessel wall interactions. Administration of bovine serum albumin as a protein control had no significant effect on leukocyte-vessel wall interactions (Figure 3.1). Intravital microscopic images of normal tissue, B16F10 tumor tissue and anginex treated tumor tissue are shown in Figure 3.2. Video images of these experiments can be viewed at <http://www.fdg.unimaas.nl/AngiogenesisLab>.

These experiments were performed in a suboptimal TNF α -induced situation in which both rolling and adhering leukocytes are present, at least in the control situation. In pilot experiments, in which no TNF α was used, leukocyte-vessel wall interactions appeared to be reduced inside a tumor as well, although the difference was smaller, and anginex also upregulated these interactions (unpublished data).

To investigate whether the effect of anginex is common among angiogenesis inhibitors, three other angiostatic drugs were tested (Figure 3.2A). Treatment of mice with endostatin (2 mg/kg/day³²) induced an increase in leukocyte-vessel wall interactions in tumor vessels as well ($p < 0.01$). Similarly, treatment of mice with angiostatin (2 mg/kg/day³³) also increased leukocyte-vessel wall interactions in tumor vessels ($p < 0.05$), although to a lesser extent than anginex and endostatin. The effects of both inhibitors were due to an increase in leukocyte adhesion in tumor vessels, whereas leukocyte rolling remained unchanged (Table 3.1). As with anginex, endostatin and angiostatin did not influence leukocyte-vessel wall interactions in the absence of a tumor (Table 3.1). TNP-470 did not induce an increase in the total level of leukocyte-vessel wall interactions in tumor vessels (Figure 3.2A). Instead, this compound tended to decrease both leukocyte adhesion and rolling and, hence, the total level of interactions (Table 3.1). This effect

Figure 3.2: Suppressed leukocyte-vessel wall interactions in tumor vessels can be normalized by angiostatic and chemotherapeutic compounds.

A. Normalization of the total number of interacting leukocytes in mouse tumor vessels to numbers observed in vessels of mice without a tumor. Data are presented as medians and interquartile ranges. Statistical significance was assessed in comparison to values in tumor vessels of untreated mice (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

B-D. Typical intravital fluorescence microscopy images of a normal ear skin vessel (B), an untreated tumor vessel (C), and an angiox treated tumor vessel (D); leukocytes are fluorescently labeled with Rhodamin 6G. The bar between B and C represents 45 μm . Video images can be viewed at '<http://www.fdg.unimaas.nl/AngiogenesisLab>'.

was seen especially in tumor-free mice, where TNP-470 significantly decreased the total level of leukocyte-vessel wall interactions ($p < 0.001$) as well as both leukocyte rolling and adhesion ($p < 0.01$; Table 3.1).

To test whether chemotherapeutic agents with claimed angiostatic properties³⁴ share the capacity to overcome tumor EC energy with specific angiogenesis inhibitors, tumor bearing mice were also treated with either paclitaxel or cyclophosphamide. Paclitaxel increased the total level of leukocyte-vessel wall interactions in tumor vessels significantly ($p < 0.05$), although interactions were still clearly lower than in healthy control vessels (Figure 3.2A). The effect of paclitaxel appeared to be due to an increase in both leukocyte adherence and leukocyte rolling (Table 3.1). In tumor-free mice, paclitaxel exhibited a dual effect by inducing an increase in leukocyte rolling ($p < 0.01$) and, at the same time, decreasing leukocyte adherence ($p < 0.001$). This resulted in a nearly significant decrease ($p < 0.07$) in the total level of leukocyte-vessel wall interactions (Table 3.1). The increase

Table 3.1: Effects of anti-angiogenesis or anti-neoplastic drugs on leukocyte-vessel wall interactions in ear skin venules of TNF α -treated C57BL/6 mice.

		Ear tumor			No tumor
		Inside tumor	Adjacent to tumor	Edge ear	
Control (no treatment)	Interacting/mm2	99 (0-161)	429 (257-610)	632 (378-1030)	758 (544-1061)
	Adhering/mm2	0 (0-0)	114 (24-356)	415 (209-549)	434 (236-720)
	Rolling/min	3 (1-6)	12 (6-20)	13 (10-17)	15 (9-24)
Anginex	Interacting/mm2	796*** (318-1030)	937* (638-1235)	965 (764-1421)	979 (518-1655)
	Adhering/mm2	168* (0-374)	371 (241-772)	503 (298-672)	332 (157-459)
	Rolling/min	9** (7-16)	16 (10-24)	26 (8-33)	23 (11-32)
Endostatin	Interacting/mm2	637** (332-1133)	693 (354-1038)	796 (474-1384)	819 (503-1222)
	Adhering/mm2	282** (0-700)	157 (0-321)	490 (163-621)	233* (92-479)
	Rolling/min	6 (2-12)	25 (10-47)	20 (8-37)	18 (11-23)
Angiostatin	Interacting/mm2	346* (35-651)	758 (355-908)	1038* (902-1664)	803 (549-973)
	Adhering/mm2	142* (0-374)	227 (138-365)	472 (101-638)	274 (99-557)
	Rolling/min	3 (1-8)	12 (5-27)	19 (13-31)	18 (15-32)
TNP-470	Interacting/mm2	0 (0-245)	249 (109-484)	442 (265-872)	152*** (84-345)
	Adhering/mm2	0 (0-0)	0* (0-0)	277 (141-408)	205*** (55-236)
	Rolling/min	1 (1-1)	7 (3-13)	9* (7-11)	5** (3-8)
Paclitaxel	Interacting/mm2	296* (150-576)	332 (263-454)	438* (290-648)	571 (434-860)
	Adhering/mm2	155* (0-297)	138 (109-297)	367 (216-463)	199*** (94-354)
	Rolling/min	8** (5-13)	30** (24-40)	25*** (21-39)	22** (12-42)
Cyclophosphamide	Interacting/mm2	199 (158-239)	265 (166-435)	364* (315-583)	303*** (133-411)
	Adhering/mm2	187* (0-239)	138 (0-466)	364 (233-599)	277* (133-407)
	Rolling/min	0* (0-1)	1*** (1-3)	1*** (1-4)	1*** (0-1)

Data are presented as median values and interquartile ranges in parentheses; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as compared to the control group;

number of vessels per category: control 10-47, anginex 12-24, endostatin 12-24, angiostatin 14-24, TNP-470 4-10, paclitaxel 11-49, cyclophosphamide 11-23

in interactions that is observed inside a tumor is, therefore, particularly striking.

Treatment of mice with cyclophosphamide also induced an increase in leukocyte-vessel wall interactions in tumor vessels (Figure 3.2A), but this effect did not reach the level of significance ($p < 0.095$). This effect of cyclophosphamide resulted from an increase in leukocyte adherence ($p < 0.05$), whereas leukocyte rolling appeared to be decreased ($p < 0.05$; Table 3.1). In tumor-free mice, treatment with this compound clearly reduced both leukocyte rolling ($p < 0.001$) and adhesion ($p < 0.05$) and, hence, also the total level of interactions ($p < 0.001$; Table 3.1). Therefore, as with paclitaxel, the non-significant increase in leukocyte-vessel wall interactions in tumor vessels after treatment with cyclophosphamide is remarkable.

Leukocyte adhesion molecule expression, systemic leukocyte counts and fluid dynamic parameters

The decrease in leukocyte-vessel wall interactions in tumor vessels cannot be explained by altered

adhesion molecule expression on leukocytes¹². We also found that the effects of the angiogenesis inhibitors were not due to changes in leukocyte adhesion molecule expression. Differences in expression of VLA-4, L-selectin, LFA-1 α , and LFA-1, on the circulating leukocytes were not observed between the different treatment groups (data not shown).

The number of leukocyte-vessel wall interactions can also be influenced by the level of leukocyte delivery to the observed blood vessels. This parameter depends on leukocyte concentration in the passing blood and on local blood flow. Treatment with anginex, endostatin, angiostatin and paclitaxel did not influence the number of circulating leukocytes or the percentage of polymorphonuclear (PMNs) and monomorphonuclear cells (MMNs; Table 3.2). TNP-470 had no effect on the systemic leukocyte count as well, although the percentage of PMNs was increased. Treatment with cyclophosphamide, however, induced a significant decrease in systemic leukocyte count ($p < 0.01$) with a concomitant decrease in the amount of PMNs ($p < 0.01$).

In all treatment groups, local vessel diameters and blood flow were not influenced (data not shown). Therefore, it is suggested that the observed effects of all angiogenesis inhibitors except cyclophosphamide on leukocyte-vessel wall interactions in and around the tumor cannot be explained by changes in leukocyte delivery to the vessels. In the present mouse model, cyclophosphamide treatment appears to be associated with a decrease in leukocyte delivery to vessels due to a significant reduction in systemic leukocyte concentration.

Table 3.2: Effect of treatment with anti-angiogenesis or anti-neoplastic drugs on systemic leukocyte concentration and percentage of polymorphonuclear (PMN) and monomorphonuclear cells (MMN) in blood of TNF α -treated C57BL/6 mice.

	Systemic leukocyte count ($\times 10^6/\text{ml}$)	PMNs (%)	MMNs (%)
Control (no treatment)	6.6 $\pm$ 0.9	16 $\pm$ 2	84 $\pm$ 2
Anginex	9.0 $\pm$ 0.8	17 $\pm$ 1	83 $\pm$ 1
Endostatin	6.0 $\pm$ 0.2	12 $\pm$ 2	88 $\pm$ 2
Angiostatin	7.7 $\pm$ 1.4	16 $\pm$ 3	84 $\pm$ 3
TNP-470	6.5 $\pm$ 1.0	33 $\pm$ 4*	67 $\pm$ 4*
Paclitaxel	5.5 $\pm$ 0.9	22 $\pm$ 3	78 $\pm$ 3
Cyclophosphamide	2.1 $\pm$ 0.3*	5 $\pm$ 2*	95 $\pm$ 2*

Data are presented as means $\pm$ SEM; * $p < 0.01$ as compared to control.

Anti-angiogenesis treatment prevents downregulation of endothelial ICAM-1 and VCAM-1 expression

To investigate whether the effects of the angiogenesis inhibitors are due to a normalization of the suppressed adhesion molecule expression on tumor ECs, *in vitro* experiments were performed using cultured b.END5 mouse EC. When cultured in the presence of 50 ng/ml bFGF, the proliferation rate of b.END5 cells is enhanced and ICAM-1 and VCAM-1 expression is downregulated by 60% and 81%, respectively (Figure 3.3A and B). Comparable but less pronounced results were observed by culturing in the presence of VEGF (data not shown). The bFGF-mediated downregulation of ICAM-1 was also observed in HUVEC (Figure 3.3C), which is in agreement with previous observations^{6,7}.

Figure 3.3: Growth factor (bFGF) induced downregulation of adhesion molecule expression on mouse EC; normalization by angiostatic and chemotherapeutic agents.

A,B. FACS analysis of ICAM-1 and VCAM-1 expression in b.ENDS mouse EC, with or without bFGF; and after treatment with angiostatic and chemotherapeutic agents. Data are expressed as mean fluorescence intensity (MFI). C. FACS analysis of ICAM-1 expression in HUVEC. D,E,F Quantitative real-time RT-PCR analysis of the effect of treatment on expression of ICAM-1 (D), VCAM-1 (E), and E-selectin (F). Data are presented as mean number of RNA copies per 100 copies of cyclophilin RNA. For all panels, data are means ($\pm$ SEM) of four independent experiments. * $p < 0.05$ versus cells without bFGF; # $p < 0.05$ versus untreated cells.

Culture in the presence of anginex completely prevented the bFGF-mediated downregulation of both ICAM-1 and VCAM-1 at the protein level (Figure 3.3A-C) and also at the RNA level (Figure 3.3C-E, for ICAM-1, VCAM-1 and E-selectin). Endostatin treatment increased adhesion molecule expression at the RNA level (Figure 3.3D-F). Angiostatin and TNP-470, the compounds with less and no activity *in vivo* (see Figure 3.2), did not significantly influence adhesion molecule expression in b.END5 mouse EC this *in vitro* bFGF-driven model. The latter compounds did have an effect on ICAM-1 expression in HUVEC, suggesting a difference in reactivity between mouse and human endothelial cells. Paclitaxel did prevent the downregulation of ICAM-1, but not VCAM-1.

To investigate adhesion molecule levels *in vivo*, B16F10 tumor tissues were analyzed. In untreated B16F10 tumors, low mRNA expression levels of both ICAM-1 and VCAM-1 were detectable whereas the expression of E-selectin was virtually absent (Figure 3.4A). In lung, the expression of all three adhesion molecules was over 100-fold higher as compared to expression in tumors (data not shown). Interestingly, in tumors of mice treated with anginex the expression of the EC specific molecules VCAM-1 and E-selectin was significantly upregulated by the treatment [2.38-fold ($p<0.05$) and 2.43 fold ($p<0.05$), respectively; Figure 3.4B]. When the change in microvessel density is also taken into account (a 3.3-fold decrease as compared to control, see Figure 3.5F) the relative increase in expression of both VCAM-1 and E-selectin is over 8-fold. The enhancement of ICAM-1 expression was not detectable using this method, most likely because this adhesion molecule is also expressed by tumor and/or stromal cells which are not affected by specific anti-angiogenesis treatment.

Figure 3.4: Effect of treatment with anginex on adhesion molecule expression in tumor tissue *in vivo*. A. Expression levels of ICAM-1, VCAM-1, and E-selectin in non-treated tumor tissues by quantitative real-time RT-PCR analysis, normalized to cyclophilin expression. B. Effect of anginex on ICAM-1, VCAM-1, and E-selectin expression in tumor tissue. Data are presented as average relative fold induction. Expression in untreated tumors was set to 1.

* $p<0.05$ as compared to untreated control tumors.

Leukocyte infiltration is enhanced by angiogenesis inhibition

We also investigated in two different mouse tumor models whether the enhancement of endothelial adhesion molecules and leukocyte-vessel wall interactions contributes to an increased leukocyte infiltration in a tumor. After a two-week period of treatment of human LS174T bearing Swiss nu/nu mice with anginex, tumor growth ($p<0.05$, Figure 3.5A) and microvessel density ($p<0.0005$, Figure 3.5B) were significantly reduced. At the same time, anginex treatment enhanced the number of infiltrating leukocytes in the tumor over 2-fold ($p<0.01$), as determined using the pan-leukocyte marker CD45 (Figure 3.5C). Similar but more pronounced results were observed in the B16F10 melanoma model in immunocompetent C57BL6 mice (Figure 3.5D-G). Treatment of mice with anginex at 6 mg/kg/day resulted in a dose dependent inhibitory effect on tumor growth. The inhibition of angiogenesis, resulting in a 3.3-fold decrease in microvessel density, caused a 6.7-fold increase in infiltrated leukocytes (Figure 3.5D and G). It was interesting to see that in this immunocompetent model also cognate immunity (cytotoxic T lymphocytes, CD8) was directed to the tumor as well (4.4-fold increase, Figure 3.5D and H).

Figure 3.5: Relationship between angiogenesis inhibition and leukocyte infiltration.

Tumor size (A), microvessel density assessment by CD31 staining (B) and infiltration by CD45+ leukocytes (C) of LS174T human colon carcinoma with and without anginex treatment. D. Immunohistochemical images of microvessel density (CD31), and infiltration by CD45+ leukocytes and CD8+ T lymphocytes in B16F10 melanoma of control and anginex treated mice. Bar in upper left panel represents 50 µm. E-H: Effect of increasing doses of anginex (2, 6, 12 mg/kg/day) on tumor size, microvessel density, total leukocyte (CD45), and cytotoxic T lymphocyte (CD8) infiltration in B16F10 melanoma. * $p<0.01$, ** $p<0.001$ and *** $p<0.0001$ as compared to control.

DISCUSSION

The present study demonstrates in an orthotopic syngeneic mouse model and in a human xenograft model that anti-angiogenesis treatment can overcome the suppression of endothelial adhesion molecules in tumors, leading to the amelioration of leukocyte-vessel wall interactions and to an increased inflammatory infiltrate in tumors.

It is known that tumors have the capacity to prevent the formation of a proper anti-tumor immune response by downregulation of endothelial adhesion molecules that are necessary for leukocyte-vessel wall interactions^{11,12,35}. This regulation is mediated by angiogenic growth factors^{6,7}. It was recently demonstrated in ovarian carcinoma patients that the expression of VEGF in tumor tissue is negatively correlated with the presence of infiltrating T lymphocytes, which was found to be the strongest prognostic factor in ovarian carcinoma³⁶. Similarly, we showed that the characteristic intense leukocyte infiltration in medullary breast carcinoma, as compared to ductal breast carcinoma, can be explained by their difference in angiogenic potential³⁷. We hypothesized that when angiogenesis eradicates the infiltration of leukocytes, inhibition of angiogenesis would circumvent tumor escape from immunity and would lead to increased leukocyte infiltration. Previously, we showed *in vitro* that the angiogenesis inhibitors platelet factor-4 (PF4) and thrombospondin-1 not only prevent the downregulation of adhesion molecules by bFGF or VEGF, but also induce re-expression of these molecules in cultured human umbilical vein derived endothelial cells³⁸. Similarly, angiostatin was found to enhance the expression of E-selectin³⁹, while the synthetic angiogenesis inhibitor SU6668 reversed the bFGF-mediated downregulation of ICAM-1, VCAM-1 and E-selectin⁴⁰. Here, we have demonstrated that this phenomenon is shared by more angiogenesis inhibitors and that it has impact on *in vivo* leukocyte-vessel wall interactions and the formation of an immune infiltrate.

In the present study, we show *in vivo* as well as *in vitro*, both at the mRNA and protein level, that angiostasis leads to re-expression of adhesion molecules on endothelial cells. The major role of ICAM-1 in leukocyte extravasation⁴¹ and the marked upregulation of ICAM-1, VCAM-1 and E-selectin in response to angiogenesis inhibition does not rule out a role for other adhesion structures. CD31, CD34 and ICAM-2, molecules with important functions in leukocyte-vessel wall interactions, are also described to be suppressed in tumor vessels^{7,42}. The same holds true for P-selectin, a molecule involved in leukocyte and platelet binding⁴³. It is expected, but remains to be proven, that these molecules are also part of angiogenesis-associated regulation.

The present results indicate that not all angiogenesis inhibitors tested showed the same inflammatory response. For treatment of cancer, the agent of choice would presumably combine the strongest vessel growth inhibitory activity with the most potent inflammatory properties. Apparently, these properties are not interdependent; e.g. TNP-470 and anginex differ largely in immune modulatory activity (see Figures 4.2 and 4.3), while they are equipotent angiogenesis and tumor growth inhibitors in the B16F10 melanoma model¹⁷, which suggests that the mechanisms of these effects are different. Anginex increased the expression of endothelial adhesion molecules on tumor endothelial cells both at mRNA and protein level. As a consequence, anginex significantly increased leukocyte-vessel wall interactions in tumor vessels to levels found in healthy control animals. This, in turn, led to increased infiltration of leukocytes into the tumor. Interestingly, angiogenesis inhibition is also suggested to be a promising approach for the treatment of diseases with a chronic inflammatory component, such as rheumatoid arthritis and psoriasis⁴⁴, in which a concomitant pro-inflammatory activity is undesirable. It is tempting to speculate that the good results with TNP-470 in arthritis models are due to the lack of pro-inflammatory activity. It might be important, therefore, to screen a larger array of compounds for this activity in order to better judge applicability in various diseases.

Interestingly, the inflammatory activity of angiostatic compounds is also shared with certain chemotherapeutic agents. It has been observed previously that chemotherapeutic compounds such as cyclophosphamide and paclitaxel can induce leukocyte infiltration in tumors⁴⁵. Currently, there is an ongoing discussion concerning the intrinsic angiostatic activity of several chemotherapeutic drugs⁴⁶. We and others have demonstrated for the tubulin binding compounds (taxanes and the vascular targeting agent combrestastatin) that an intrinsic anti-angiogenesis activity exists at the level of endothelial migration and tube formation, at concentrations that do not affect cell growth⁴⁷. The presently shown shared activity of paclitaxel and angiogenesis inhibitors at the level of adhesion molecule regulation and leukocyte-vessel wall interactions may support the theory that certain chemotherapeutics are angiostatic and may be used at ultra low dosages for prolonged periods of time as treatment of cancer.

The effects of the angiogenesis inhibitor TNP-470 on leukocyte-vessel wall interactions and adhesion molecule expression contrasted with the results obtained with the specific angiogenesis inhibitors anginex, endostatin and angiostatin. This may be explained by the fact that TNP-470 has other indirect effects, which is also suggested by the systemic decrease in leukocyte-vessel wall interactions in tumor-free mice (Table 3.1) and the change of leukocyte composition in peripheral blood (Table 3.2). Like TNP-470, cyclophosphamide also decreased leukocyte-vessel wall interactions in healthy microvessels *in vivo*, while paclitaxel only reduced the level of leukocyte adhesion in these vessels. This decrease in leukocyte-vessel wall interactions in a tumor-free environment may mask the upregulating effect of these agents on leukocyte-vessel wall interactions in tumor vessels.

Here, we used recently developed tumor models¹² in which leukocyte-vessel wall interactions can be measured non-invasively, allowing longitudinal analysis during tumor development and over the course of anti-cancer treatment. The combined assessment of leukocyte-vessel wall interactions inside the vascular system and leukocyte infiltration into the tissue provides a functional meaning to changes in leukocyte adhesion. Our findings indicate that the increase in leukocyte-vessel wall interactions in tumor vessels is followed by increased infiltration of leukocytes into the tumor tissue, where they can help to suppress tumor growth. It is an interesting observation that both angiogenesis inhibitors and chemotherapeutic compounds can elucidate this effect. It is tempting to speculate that the reported, sometimes very good, results from combining both treatment strategies^{18,48} is explained by this enhancement of inflammatory infiltrate. In a preliminary pilot study we found that combination of paclitaxel and anginex leads to an enhanced leukocyte infiltrate as compared with either paclitaxel or anginex alone (unpublished results). It remains to be elucidated, however, which leukocyte subtypes are involved in increased homing to the tumor.

The present study was focused on investigating the escape of tumors from immunity and how the leukocyte infiltrate can be augmented in tumors. Although the beneficial effect of cognate anti-tumor immunity is not debated at all, it should be kept in mind that several studies have shown that leukocytes, by their inflammatory cytokine/chemokine production, and by their release of matrix metalloproteinases, can contribute to angiogenesis programs and thereby to tumor progression as well^{49,50}. Tumor associated macrophages have been mostly recognized for this phenomenon⁵¹. We, however, favor the view that leukocyte infiltration should be promoted in order to drive anti-tumor immunity. In conclusion, the present study demonstrates that angiogenesis inhibitors increase leukocyte-vessel wall interactions in tumor vessels, which leads to increased leukocyte infiltration into tumor tissue. This anti-tumor inflammatory effect of angiogenesis inhibitors is mediated by increased expression of adhesion molecules on tumor endothelial cells, and not by altered expression of leukocyte adhesion molecules, effects on the amount of circulating leukocytes, or other direct or indirect vascular effects modulating fluid dynamic parameters. These data strongly support the concept of combining modern

immunotherapeutic approaches with angiostatic therapies. At least additive and hopefully synergistic anti-tumor responses would be expected from this combination.

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CHAPTER 4

HIV-1 Tat increases the adhesion of monocytes and T-cells to the endothelium *in vitro* and *in vivo*: implications for AIDS- associated vasculopathy

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ABSTRACT

HIV-1 infected patients exhibit severe damages of the aortic endothelium, develop angioproliferative lesions such as Kaposi's sarcoma (KS), and have an increased risk of cardiovascular diseases and atherosclerosis. An increased adhesion of leukocytes to the endothelium is a common pathogenic parameter of AIDS-associated vascular diseases. Here we show that the HIV-1 Tat protein, a regulatory protein of HIV-1 released by infected cells, and $\text{TNF}\alpha$, a cytokine increased in sera and tissues of HIV-1-infected patients, activate synergistically the adhesion of leukocytes to endothelial cells both *in vitro* and *in vivo*. This effect is selectively mediated by HIV-1 Tat, since HIV-1 Nef, another HIV-1 regulatory protein, and the HIV-1 envelope protein gp41, had no effect. *In vitro* adhesion assays with PBMC and quantitative cell type analysis of adherent cells by FACS demonstrated that HIV-1 Tat selectively activates the adhesion of T-cells and monocytes but not of B-cells. Intravital microscopic studies in mice confirmed the synergistic activity of HIV-1 Tat and $\text{TNF}\alpha$ on leukocyte adhesion to the endothelium *in vivo*. These data indicate that HIV-1 Tat in cooperation with $\text{TNF}\alpha$ may contribute to the vascular damage and cardiovascular diseases observed in AIDS patients but also to the prominent extravasation of T-cells and monocytes which is a key process in the formation and progression of KS lesions.

INTRODUCTION

HIV-1 infection is accompanied by vascular diseases. Until recently, the clinically most evident manifestation of AIDS-associated vasculopathy was Kaposi's sarcoma (AIDS-KS), an angio-proliferative tumor with prominent infiltration of monocytes and T-cells¹. In the era of the highly active antiretroviral therapy (HAART), also other diseases have been recognized that indicate chronic vessel activation and dysfunction in HIV-1 infection. For example, cardiovascular diseases account for up to 10% of the current death cases in HIV-1-infected patients², evidence of atherosclerosis has been found in 75% of dyslipidemic HIV-1 patients³, and an increasing frequency of pulmonary hypertension has been reported⁴. These findings are in agreement with earlier observations by us demonstrating severe alterations of the structural integrity of the aortic endothelium of HIV-1-infected patients, concomitant with an increased adhesion of leukocytes⁵.

HIV-1 Tat is a transcriptional activator of viral gene expression produced early after infection and is essential for virus replication⁶⁻⁸. During acute infection of T-cells by HIV-1, HIV-1 Tat is released from the cells in an active form and via a leaderless secretory pathway⁹⁻¹¹. Consistent with these observations, HIV-1 Tat has been detected in sera of HIV-1-infected individuals¹² and in AIDS-KS lesions particularly in vessels^{13,14}.

Extracellular HIV-1 Tat released by infected cells can enter infected or non-infected cells and can activate a variety of genes regulated by specific viral and cellular promoters¹⁵⁻¹⁷. Of note, HIV-1 Tat also increases angiogenesis mediated by bFGF and is capable of inducing the growth, migration and invasion of endothelial cell-like cultures derived from KS lesions¹⁸⁻²² and of normal endothelial cells that have been activated by inflammatory cytokines (IC) including TNF α ²³⁻²⁶. This is due to the HIV-1 Tat molecular mimicry of extracellular matrix proteins and is mediated by two regions of the protein. The basic region that retrieves bFGF from heparan sulfate proteoglycans of the extracellular matrix²⁷⁻²⁹ and the RGD region that binds to the $\alpha 5 \beta 1$ and $\alpha v \beta 3$ integrins³⁰ and through this mediates cell adhesion, migration and cell invasion, which in turn is associated with the induction of matrix metalloproteinase (MMP)-1 by HIV-1 Tat^{31,32}. In addition, HIV-1 Tat induces the cell surface expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial leukocyte adhesion molecule-1 (E-selectin) in endothelial cells³³, suggesting that it may induce the interaction of endothelial cells with leukocytes. In fact, it has been shown that HIV-1 Tat enhances the migration and the invasion of monocytes into reconstituted basement membranes³⁴. In addition, HIV-1 Tat up-regulates the expression of, 2-integrins in these cells and increases their adhesion to TNF α -treated endothelial cell monolayers³⁵. The latter is accompanied by a substantial disruption of the endothelial monolayer and it has been proposed that a HIV-1 Tat-induced up-regulation of MMP-9 may contribute to this effect³⁵. The stimulation of monocyte adhesion has been attributed to the induction by HIV-1 Tat of autocrinely active cytokines, namely IL-1 and TNF α ³⁶.

All the effects of HIV-1 Tat on endothelial cells and their interaction with monocytes are significantly increased by or require a previous exposure of the cells to IC, such as IFN γ , IL-1, and TNF α . Of note, these same cytokines are present at increased concentrations in the sera of HIV-1-infected patients and in both sera and tissues of patients with KS or at risk of KS³⁷⁻³⁹. In particular, IL-1, and IFN γ are more closely associated with acute infection⁴⁰⁻⁴², whereas increasing serum concentrations of TNF correlate with disease progression⁴³⁻⁴⁵.

Here we show that TNF α and HIV-1 Tat increase synergistically the adhesion of leukocytes to endothelial cells *in vitro* and that this effect is selectively mediated by HIV-1 Tat and not by other HIV-1-encoded proteins, including the core protein gp41 and HIV-1 Nef, another regulatory factor of HIV-1. Further, adhesion experiments with PBMC and FACS analysis of the adherent cells

demonstrated that HIV-1 Tat selectively increases the adhesiveness of monocytes and T-cells but not of B-cells. Finally, an intravital microscopic study in mice demonstrated for the first time that HIV-1 Tat and TNF α cooperate in the activation of leukocyte adhesion to the endothelium also *in vivo*.

MATERIALS AND METHODS

Recombinant HIV-1 proteins

Tat from HIV-1 type IIIB-BH-10 (subtype B) was expressed in *E. coli* and purified to homogeneity by heparin-affinity chromatography and HPLC, as described⁴⁶. The purified HIV-1 Tat protein was fully biologically active, as tested by various assays, including rescue assays on the HLM-1 cell line carrying a Tat-defective HIV-1 provirus, or the induction of chloramphenicol acetyl transferase activity in HL3T1 cells containing a HIV-1-long terminal repeat chloramphenicol acetyl transferase construct and by uptake studies with dendritic cells⁴⁷. To prevent oxidation, the HIV-1 Tat protein was stored lyophilized at -80°C and reconstituted in degassed buffer before use⁴⁸. To prevent attachment of the protein to surfaces, plastic tips and vials before use were rinsed in 0.1% PBS-BSA or in RPMI 1640 (Invitrogen, Karlsruhe, Germany) supplemented with 20 mM HEPES (Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine (Invitrogen) and 15% fetal bovine serum (FBS) (Invitrogen). In addition, because HIV-1 Tat is also photo- and thermosensitive^{49,50}, the handling of the protein was always performed in the dark and on ice. In all cases endotoxin concentration was below the detection limit (<0.05 EU/ μ g), as determined by the Limulus Amoebocyte Lysate analysis (Pyrochrome, Associates of Cape Cod, Falmouth, MA).

Nef from HIV LAV-1 Bru was a gift from V. Erfle (GSF-National Research Center for Environment and Health, Germany). It was purified as described in detail by Kohleisen et al.⁵¹ and handled as described above for HIV-1 Tat.

Since gp41 is rather hydrophobic, a recombinant fusion protein between maltose-binding protein (MBP) and an 82-amino-acid-long extracellular region (amino acids 565 to 647) of gp41 was used for the experiments (Intracel, Cambridge, MA). To rule out possible side effects of MBP, recombinant MBP (New England Biolabs, Beverly, MA) was used as the negative control⁵².

Cell Cultures

PBMC were isolated from fresh blood of three different healthy donors by Percoll (Amersham Biosciences, Freiburg, Germany) density gradients ($d = 1.080$) at 800 x g for 30 min. The PBMC were collected at the interface and washed twice with PBS. Then the cells were suspended at a concentration of 5×10^5 cells/ml and maintained in RPMI 1640 containing 5% FBS. The U937 monocytic cell line was obtained from ATCC and was grown in RPMI 1640-10% FBS.

HMVEC were obtained from Bio Whittaker (Verviers, Belgium) and maintained further in endothelial basal medium (EBM-2 MV, Bio Whittaker) supplemented with 5% FBS as has been described^{53,54}.

Cell adhesion assay

Cell adhesion experiments were carried out as described⁵⁵. HMVEC were seeded at 7×10^4 cells/well in eight-well microchamber slides (Nunc, Wiesbaden, Germany) and incubated overnight in EBM-2.5% FBS. Human recombinant TNF α (Roche Diagnostic, Mannheim, Germany) was added in EBM-0.5% FBS for 6 h at the concentrations indicated in the Figures. Subsequently, 500 μ l of a suspension of U937 cells (5×10^5 cells/ml) or of PBMC (1×10^6 cells/ml) were added, and after

15 min non-adherent cells were gently removed by dipping the slides into RPMI 1640-1% FBS. Afterwards, the cells were fixed (30 min, RPMI 1640-1% FBS-5% glutaraldehyde) and the adherent cells were counted using a photo-imaging system (Optimas, Stemmer Imaging, Puchheim, Germany). Each experiment was carried out in triplicate and 8 different optical fields were counted of each well. Results are expressed as the mean ($\pm$ SD) of three independent experiments. Statistical significance was assessed using the student's *t*-test. $P \leq 0.05$ was regarded as statistically significant.

For FACS analysis HMVEC were seeded at 1×10^6 cells in culture dishes (8 cm²) and, as described above, incubated overnight in EBM-2.5% FBS and stimulated in EBM-0.5% FBS containing TNF α for 6 h. Afterwards 2 ml of a suspension of PBMC (1×10^6 cells/ml) were added, and after 15 min non-adherent cells were removed by gentle washing of the cells twice with PBS. Subsequently the adherent cells were harvested by treatment with Accutase (PPA Laboratories, Coelbe, Germany) and re-suspended in PBS-1% BSA-0.01% NaAz. Then the cells (1×10^6 cells/100 μ l) were incubated with FITC-conjugated anti-CD45 mAb (5 μ g/ml, IgG1), anti-CD3 mAb (5 μ g/ml, IgG1), anti-CD20 mAb (5 μ g/ml, IgG1) or PerCP-conjugated anti-CD14 mAb (0.5 μ g/ml, IgG2b) for 30 min at RT. To prove staining specificity the cells were incubated for 30 min at RT with FITC-conjugated IgG1 (5 μ g/ml) and PerCP-conjugated IgG2b (5 μ g/ml) control antibodies. All mAbs were purchased from BD Biosciences (Heidelberg, Germany). After washing the cells twice they were fixed with 2% paraformaldehyde and analysed (10,000 events/measurement) with a FACSCalibur (BD Biosciences). The data were processed using the CellQuest programm (BD Biosciences). Experiments were repeated 6 times and the results are presented as the mean ($\pm$ SD). Statistical significance was assessed using the student's *t*-test. $P \leq 0.05$ was regarded as statistically significant.

Intravital microscopy

The experiments were approved by the Ethical Committee on Animal Experiments of Maastricht University (The Netherlands). Intravital microscopy was performed with C57Bl/6 mice of a body weight varying from 25 g to 30 g. On the day of the experiments the mice were injected i. p. with either 100 ng ($n = 3$) recombinant murine TNF α (R&D Systems, Abingdon, England), 20 μ g HIV-1 Tat ($n = 3$), 20 μ g HIV-1 Tat and 100 ng recombinant murine TNF α ($n = 2$) or PBS-0.1% BSA ($n = 2$) as a control. Four hours later the mice were anesthetized by subcutaneous injection of a mixture of ketamine [0.1 mg/g body weight (b.w.), Nimatek, Ad Usem Veterinarium, Cuijk, The Netherlands] and xylazine (0.02 mg/g b.w., Sedamun, Ad Usem Veterinarium, Cuijk). To enable intravital microscopic fluorescence-based observation of leukocytes, 10 - 20 μ l Rhodamine-6G (Sigma-Aldrich) dissolved in NaCl 0.9%-solution (1 mg/ml) and filtered through a 0.22 μ m filter (Millipore, Etten-Leur, The Netherlands) were injected through a PE-10 catheter (Portrex, Kent, England) into the tail vein. Ear skin venules in both ears were visualized using a Leitz intravital microscope carrying a Leitz x 1.25 Ploemopak 2.2 for interchangeable filter sets and a Leitz x 25 salt water immersion objective (numerical aperture 0.60). To this purpose, the mouse was positioned on a preheated platform (37 °C), the ear was placed on a glass ring with the dorsal side up and covered with a drop of paraffin oil and a cover slip. Trans-illumination was performed using a 60 W tungsten lamp, while for fluorescence microscopy a Leitz N2.1 filter set (excitation filter BP 515-560, dichroic mirror RHP 580, and barrier filter LP580) was used in combination with a 100 W mercury lamp. Images were projected on a charge coupled device camera (Hamamatsu Photonics, C2400, Hamamatsu City, Japan), coupled to an intensifier unit (Hamamatsu Photonics A4405), and recorded on videotape (sVHS recorder, Panasonic, Japan) for off-line analysis. The final optical magnification at the front plane of the camera was x 40. In all experiments the mid-plane of a vessel was kept in focus.

Throughout the experiment, body temperature was kept at 37 °C with an infra-red heating lamp controlled by a thermo-analyzer system connected to a rectal probe.

Experimental parameters: Venules were selected within the diameter range of 15–45 µm. Leukocyte-vessel wall interactions were analyzed as described²⁹. In short, leukocytes were considered as rolling when their velocity along the vessel wall was at least 10 fold lower than that of the free flowing blood cells. The level of leukocyte rolling was measured off-line by counting in duplicate the number of cells that rolled through a predefined segment of the vessel during a period of 120 seconds. Data were expressed as the number of rolling cells passing a vessel segment per minute.

The level of leukocyte adhesion was assessed in a 100 µm segment of the venule and expressed as the number of cells per endothelial surface area. Leukocytes were considered adherent when they remained stationary for at least 30 seconds.

Statistics: Because of their non-symmetrical distribution, data are presented as medians with interquartile ranges (i.e. the spread from 25th to 75th percentile). Differences between two independent data groups were tested with the Mann-Whitney U-test.

Results

HIV-1 Tat and TNF α activate synergistically the adhesion of U937 cells and PBMC to endothelial cells

In vitro adhesion assays were performed with primary human microvascular endothelial cells (HMVEC) and the promonocytic cell line U937. Activation of HMVEC with sub-optimal concentrations of TNF α (0.5 ng/ml) resulted in a 3-fold increase of U937 cell adhesion to endothelial cells (Figure 4.1A, ctrl., compare white and black columns). Incubation of U937 cells with increasing concentrations of the HIV-1 Tat protein stimulated the adhesion of these cells to HMVEC by 1.7 to 2-fold (Figure 4.1A, white columns). Simultaneous stimulation of U937 cells with HIV-1 Tat (10 ng/ml and 100 ng/ml) and HMVEC with TNF α (0.5 ng/ml) resulted in a strong synergistic increase (10-fold) of U937 cell adhesion to HMVEC (Figure 4.1A, middle and right black columns). The differences of the results obtained by stimulation with either TNF α or HIV-1 Tat alone as compared to the simultaneous stimulation with both factors were significant ($P \leq 0.007$ (10 ng/ml HIV-1 Tat), $P \leq 0.003$ (100 ng/ml HIV-1 Tat)).

Adhesiveness of U937 cells to TNF α -stimulated endothelial cells was significantly ($P \leq 0.01$) and selectively activated by the HIV-1 Tat protein but not by the HIV-1 core protein gp41 or by Nef, another regulatory protein of HIV-1 (Figure 4.1B). Similarly to U937 cells, also adhesion of PBMC was significantly ($P \leq 0.03$) and selectively increased (1.6 fold) by HIV-1 Tat but not by gp41 and by Nef (Figure 4.1C).

HIV-1 Tat preferentially activates adhesion of primary monocytes and T-lymphocytes to HMVEC

In order to examine which cell type (B-cells, T-cells or monocytes) present in PBMC is preferentially activated by HIV-1 Tat to adhere to the endothelium, adhesion experiments were performed with PBMC. Leukocytes bound to endothelial cells were stained with different cell-type associated markers and quantified by FACS analysis (Figures. 5.2 and 5.3).

TNF α treatment of HMVEC increased the numbers (within 10,000 counted cells) of adherent CD45⁺ leukocytes (Figure 4.2A versus B), CD3⁺ T-cells (Figure 4.2E versus F), CD20⁺ B-cells (Figure 4.2G versus H) and CD14⁺ monocytes (Figure 4.2I versus K). The relative number of CD45⁺ endothelial cells was decreased (Figure 4.2C versus D), due to increased numbers of CD45⁺ cells

Figure 4.1

HIV-1 Tat and TNF α increase synergistically the adhesion of leukocytes to HMVEC *in vitro*. *In vitro* adhesion of U937 cells (A,B) and PBMC (C) to HMVEC. (A) HMVEC were either non-stimulated (white columns) or stimulated with TNF α (0.5 ng/ml, black columns). U937 cells were either non-stimulated (ctrl.) or activated with increasing concentrations of HIV-1 Tat. (*) $P \leq 0.007$ and (**) $P \leq 0.003$ for the differences of results obtained when only one cell type (U937 cells or HMVEC) was stimulated by TNF α or HIV-1 Tat, respectively as compared to TNF α -stimulated HMVEC and HIV-1 Tat stimulated U937 cells. (B) HMVEC were treated with TNF α (0.1 ng/ml). U937 cells were either treated with gp41 (182 ng/ml, left, black column), Nef (100 ng/ml, middle, black column) or HIV-1 Tat (100 ng/ml, right, black column) or were non-stimulated (white columns). (***) $P \leq 0.01$ compared to non-stimulated U937-cells. (C) The same conditions as described in (B) but PBMC were used instead of U937 cells. (****) $P \leq 0.03$ compared to non-stimulated PBMC. In each experiment stimulation of HMVEC was carried out for 6 h and stimulation of U937 cells and PBMC for 24 h. In each case the mean values and standard deviations of three different experiments are shown.

10,000 cells evaluated. Staining specificity was demonstrated with isotype matched IgG1 and IgG2b control antibodies (Figure 4.2L-O). Calculation of the number of adherent cells per 1,000 endothelial cells confirmed that TNF α treatment of HMVEC increased (2-fold) the adhesiveness to endothelial cells of all leukocyte populations examined, including T-cells ($P \leq 0.02$), B-cells ($P \leq 0.02$) and monocytes ($P \leq 0.05$) (Figure 4.2P).

The treatment of PBMC with the HIV-1 Tat protein further increased the relative numbers (within 10,000 counted cells) of CD45⁺ leukocytes (Figure 4.3A and B) adhering to the TNF α -stimulated HMVEC. However, HIV-1 Tat selectively increased the numbers of CD3⁺ T-cells (Figure 4.3E and F) and CD14⁺ monocytes (Figure 4.3I and K) adherent to endothelial cells, but not of CD20⁺ B-cells (Figure 4.3G and H). Again and in agreement with the experimental set-up the increase in leukocyte adhesion was paralleled by a relative decrease of the numbers of CD45⁺ endothelial cells (Figure 4.3C and D). Staining specificity was demonstrated with isotype matched IgG1 and IgG2b control antibodies (Figure 4.3L-O). Calculation of the number of adherent cells per 1,000 endothelial cells indicated that HIV-1 Tat increases adhesion of leukocytes by 1.8-fold ($P \leq 0.02$) (Figure 4.3P) which is consistent with the previous adhesion experiments (Figure 4.1C). Within the adherent cells the numbers of monocytes were increased 2.2-fold ($P \leq 0.02$) and those of CD3⁺ T-cells 1.8-fold ($P \leq 0.02$). In contrast, the number of adherent B-cells was not significantly increased by HIV-1 Tat stimulation (Figure 4.3P).

HIV-1 Tat and TNF α activate synergistically the adhesion of leukocytes to endothelial cells *in vivo*

To investigate the effect of HIV-1 Tat and TNF α on leukocyte adhesion *in vivo* an intravital microscopy study was performed. In the present mouse model TNF α at concentrations of 500 ng

Figure 4.2

TNF α stimulation of HMVEC increases the adhesion of T-cells, B-cells and monocytes. HMVEC that were either non-stimulated (control) or treated for 6 h with TNF α (0.1 ng/ml) were overlaid with freshly isolated PBMC. Non-adherent cells were removed by washing. Adherent cells and HMVEC were harvested, stained with FITC (FL1-H)-coupled antibodies to CD45 (A and B), CD3 (E and F), CD20 (G and H) and a PE (FL3-H)-coupled antibody to CD14 (I and K) and quantitatively determined by FACS analysis of 10,000 cells. CD45 negative cells (CD45 neg.) represent the HMVEC (C and D). To prove staining specificity the cells were incubated with isotype matched control antibodies (L-O). A representative result of three independent experiments is shown. In (P) the relative numbers of CD45+, CD3+, CD20+ and CD14+ cells that bound to 1,000 HMVEC are graphically displayed. The mean values and standard deviations were calculated from three different experimental points. (*) $P \leq 0.02$ and (**) $P \leq 0.05$ compared to unstimulated HMVEC.

significantly increases adhesion of leukocytes (Dirkx et al. 2003)57. In order to evaluate potential synergistic effects of HIV-1 Tat and TNF α sub-optimal concentrations of TNF α (100 ng) were used that still significantly ($P \leq 0.001$) increased leukocyte rolling (Figure 4.4A) but had no effect on the adhesion of leukocytes to the vessel wall (Figure 4.4B). HIV-1 Tat was also used in a concentration (20 μ g) that significantly ($P \leq 0.001$) stimulated rolling (Figure 4.4A) but only slightly stimulated cell adhesion (Figure 4.4B). The combined application of HIV-1 Tat and TNF α significantly increased adhesion of leukocytes ($P \leq 0.001$, Figure 4.4B) at the expense of the number of rolling cells (Figure 4.4A). From earlier studies it is known that the rolling of leukocytes along the endothelium is the necessary step preceding leukocyte adhesion (von Andrian et al. 1991). The present data strongly suggest that the combination of HIV-1 Tat and TNF α significantly upregulates endothelial expression

Figure 4.3

HIV-1 Tat stimulation of PBMC increases selectively the adhesion of monocytes and T-cells. Freshly isolated PBMC were either non-stimulated (control) or treated for 24 h with HIV-1 Tat (100 ng/ml) and applied onto TNF α (0.1 ng/ml, 6 h)-treated HMVEC. Non-adherent cells were removed by washing. Adherent cells and HMVEC were harvested, stained with FITC (FL1-H)-coupled antibodies to CD45 (A and B), CD3 (E and F), CD20 (G and H) and a PerCP (FL3-H)-coupled antibody to CD14 (I and K) and quantitatively determined by FACS analysis of 10,000 cells. CD45 negative cells (CD45 neg.) represent the HMVEC (C and D). To prove staining specificity the cells were incubated with isotype matched control antibodies (L-O). A representative result of three independent experiments is shown. In (P) the relative numbers of CD45+, CD3+, CD20+ and CD14+ cells that bound to 1,000 HMVEC are graphically displayed. The mean values and standard deviations were calculated from three different experimental points. (*) $P \leq 0.02$ compared to control.

of adhesion molecules, resulting in a fast transition of rolling to adhesion. This is illustrated by photographs of microvessels in the different groups (Figure 4.4C-F). In the control situation a low level of leukocyte-vessel wall interactions is observed (Figure 4.4C), which is increased after administration of TNF α (Figure 4.4D), or HIV-1 Tat (Figure 4.4E) in which case most leukocytes roll along the vessel wall. Administration of both substances also induces an increase in leukocyte-vessel wall interactions, but now most cells adhere (as indicated by the black arrows) and less are rolling (Figure 4.4F).

Figure 4.4

HIV-1 Tat and TNF α increase synergistically the adhesion of leukocytes to the endothelium *in vivo*. C57Bl/6 mice were injected *i. p.* with either TNF α (100 ng, $n = 3$), HIV-1 Tat (20 μ g, $n = 3$), a combination of both factors ($n = 2$) or with an equivalent volume of dilution medium (PBS/0.1%BSA, $n = 2$). By intravital microscopy (A) the numbers of rolling cells passing a vessel segment per minute and (B) the number of adherent leukocytes per mm² endothelial surface area were determined. Because of their non-symmetrical distribution data are presented as medians and interquartile ranges. (***) $P \leq 0.001$ compared to untreated mice. Images of intravital fluorescence microscopy are shown of ear venules in mice treated with PBS/0.1% BSA (C), 100 ng TNF α (D), 20 μ g Tat (E) and a combination of both factors (F). Adhering leukocytes are indicated by black arrows, all other leukocytes can be considered as rolling.

DISCUSSION

The endothelium of HIV-1-infected patients is characterised by increased numbers of adherent leukocytes as compared to non-infected patients⁵⁸. This may support the recruitment of lympho-/monocytes and of human herpesvirus-8 into AIDS-KS lesions^{1,59}. In addition, in the era of HAART, which significantly elongated survival times of the patients, this may contribute to the increased risk of cardiovascular diseases and atherosclerosis in HIV-1-infected individuals^{60,61}.

The HIV-1 Tat protein *in vitro* has been shown to affect the monocyte/endothelial cell interaction. HIV-1 Tat up-regulates the expression of β 2-integrin adhesion molecules and MMP-9 expression in monocytes, which increases monocyte/endothelial cell adhesion, enhances monocyte dependent damage of endothelial cell monolayers, and promotes invasive behaviour of monocytes⁶²⁻⁶⁴.

HIV-1 Tat has also been shown to be active in mice. Transgenic mice expressing HIV-1 Tat develop tumors^{65,66}, and inoculation into mice of purified HIV-1 Tat in combination with IC or bFGF can lead to the formation of vascular lesions⁶⁷⁻⁷⁰. In the present study we demonstrated for the first time that HIV-1 Tat and TNF- α can synergistically increase the adhesion of leukocytes to the endothelium *in vivo*. Although high concentrations of HIV-1 Tat (20 μ g) as compared to TNF α (100

ng) were used, it has to be considered that two features of HIV-1 Tat may quickly reduce the effective concentration of the protein in the circulation after intra-peritoneal injection. First, HIV-1 Tat with its basic sequence binds rapidly to heparan sulfate proteoglycans of the cell surface and the extracellular matrix⁷¹. Second, it has been shown that 4 h after injection in mice HIV-1 Tat is delivered to all tissues and incorporated into the cells⁷². For these reasons high concentrations of HIV-1 Tat were used. However, in HIV-1 infected patients HIV-1 Tat is highly expressed and released by infected cells and may activate leukocytes in the near proximity, possibly in lymph nodes. Activated leukocytes after re-entering the circulation may adhere with increased efficiency to the endothelium of HIV-1-infected patients that is chronically exposed to increased concentrations of IC.

Our findings may be specifically of relevance for the pathogenesis of AIDS-KS. Early stage KS lesions are characterized by an inflammatory-granulation-type reaction with activated proliferating endothelial cells forming new vessel-like (for review see^{1,73}). The nature of the inflammatory cell infiltrate of KS is important since it is the first to appear and precedes the appearance of the tumor cells, the so-called KS spindle cells. Immunohistochemical studies indicate a prevalent infiltration of monocyte-macrophages (CD68+, MAC387+) together with CD4-positive and CD8-positive T-cells (CD3+), whereas B-cells (CD19+, CD20+) are rare or absent⁷⁴⁻⁷⁹. Our findings that the co-operative activity of HIV-1 Tat and TNF α on adhesion selectively targets monocytes and T-cells is well in agreement with the leukocyte infiltration observed in AIDS-KS lesions. The co-operative activity of both factors may explain the 20.000-fold increased incidence of KS in AIDS⁸⁰ and possibly may contribute to other vascular defects in AIDS that are associated with increased leukocyte adhesion and extravasation into tissues, including cardiovascular diseases and atherosclerosis.

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CHAPTER 5

Monocyte/macrophage infiltration in tumors: modulators of angiogenesis

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ABSTRACT

The role of a tumor immune infiltrate in cancer progression and metastasis has frequently been debated. Although often considered to be associated with improved prognosis and leading to the enhanced survival of cancer patients, inflammatory cells have also been described to assist the tumor's capabilities to progress, proliferate and metastasize. Tumor associated macrophages (TAMs), for example, have shown to be symbiotically related to tumor cells: tumor cells recruit TAMs and provide them with survival factors, while TAMs in turn produce a variety of angiogenic factors in response to the tumor microenvironment. This review will describe the composition of an immune infiltrate in tumors, and the angiogenic and angiostatic properties of the cells present. Special emphasis will be on the angiogenesis-associated activities of TAMs. The development of immuno- and gene therapies using TAMs to mediate tumor cytotoxicity or to deliver gene constructs will be discussed as well. Since immunotherapy has so far not been as effective as anticipated, a combination therapy in which angiostatic agents are used as well is put forward as a novel strategy to treat cancer.

INTRODUCTION

Without the formation of new blood vessels (angiogenesis), expansion of a tumor mass is limited to 1-2 mm in size because of insufficient supply of oxygen and nutrients, a concept first proposed by Dr J. Folkman in 1971¹. Many tumors remain dormant at this size and, therefore, stay clinically undetectable for years². A dys-equilibrium between positive and negative angiogenesis regulators eventually results in the progression of these *in situ* tumors to an angiogenic phenotype, the so called angiogenic switch^{3,4}. Tumor angiogenesis is promoted by hypoxia resulting in enhanced expression of angiogenic factors such as vascular endothelial growth factor (VEGF), interleukin-8 (IL-8) and basic fibroblast growth factor (bFGF), and in decreased expression of angiogenesis inhibitors such as platelet factor-4 (PF-4), thrombospondin and angiostatin⁵.

Once a tumor is vascularized it is infiltrated by leukocytes, a phenomenon observed in all solid cancers. The first observation of leukocyte infiltration in tumors was done by Virchow in 1863⁶ and was thought to be the result of chronic inflammation that was already present before tumor development. Nowadays, however, it is known that the presence of leukocytes is a consequence of an immune reaction to the tumor itself -first innate and later specific immunity-, since the immune system is able to recognize tumor-associated antigens⁷. In cancer patients, specific cytotoxic T-lymphocytes recognizing tumor antigens have been reported and the presence of these cells is related to a better prognosis. In addition, antibodies to tumor-associated antigens produced by B-cells may also play a role in limiting tumor growth^{8,9}. Leukocyte infiltration in tumors is, therefore, often associated with better prognosis and overall survival⁷. Moreover, the ability of the immune system to recognize antigens expressed by tumors or tumor microvasculature can be used to identify molecules to define targets for cancer immunotherapy⁸.

Under normal physiological circumstances, leukocytes are recruited in response to inflammation by the local synthesis of chemokines and other cytokines and by products that are associated with tissue breakdown. These processes are all part of a complex signalling system including recognition of the pathological state, organisation of a proper cellular response and subsequent suppression of this response once the situation is resolved. In wound healing, these processes include proliferation and migration of epithelial cells, angiogenesis and tissue remodelling¹⁰. In tumors, similar chemoattractive factors are thought to play a role in leukocyte recruitment. Tumor cells, however, continuously send out signals that are associated with growth and migration processes, which recruit additional leukocytes to the tumor site¹¹. This concept has contributed to the characterization of tumors as "wounds that fail to heal"¹².

Although associated with better prognosis, immune responses to a tumor are often weak and not able to completely destroy a tumor⁷, suggesting that tumors have developed mechanisms to escape immune surveillance. Among a series of mechanisms that can explain this escape, including tolerance induction and activation of regulatory immune functions¹³, one mechanism to escape tumor-directed immunity is inhibition of leukocyte-vessel wall interactions in tumor vessels¹⁴⁻¹⁷, due to suppressed expression of endothelial adhesion molecules¹⁸⁻²¹ and induction of endothelial cell anergy²². As a result, tumors frequently show only limited leukocyte infiltration, predominantly by cells of the innate immune system¹⁹. Leukocytes that do reach the tumor, often remain localized in the tumor periphery or stroma and are often not able to exert strong antitumor effects¹². Moreover, it is known that infiltrated leukocytes show impaired maturation due to exposure to the tumor microenvironment^{7,23}. Therefore, infiltrated leukocytes are often not capable of destroying the tumor completely. By contrast, some leukocyte subsets have been described to promote proliferation and metastasis and, hence, tumor development. Tumor associated macrophages (TAM), for example, can exert protumor effects through the secretion of immunosuppressive cytokines, the release of free

radicals like nitric oxide (NO) and hydrogen peroxide (rendering immune cells hyporesponsive or even apoptotic) and, last but not least, the secretion of angiogenic cytokines and enzymes²⁴. This review will focus on the angiogenic effects of leukocytes that are infiltrated in a tumor. It will discuss the composition of a leukocyte infiltrate in tumors, and the angiogenic and angiostatic properties of the immune cells present. Special emphasis will be on the role of tumor associated macrophages, their capability to reach a tumor in large quantities and, hence, their possible role in immunotherapy.

IDENTITY AND ANGIOGENESIS RELATED EFFECTS OF CELLS IN A TUMOR IMMUNE INFILTRATE

In tumor immune infiltrates, practically all cells involved in inflammation can be found. Leukocytes are known to accumulate in a tumor through a series of interactions with tumor endothelial cells, including leukocyte tethering, rolling, firm adhesion and eventually diapedesis²⁵. The notion that tumor infiltration by leukocytes is always a prognostically positive phenomenon has changed in recent years; the finding that angiogenic factors cannot only be produced by tumor cells, but also by infiltrating leukocytes indicates possible stimulating effects on tumor angiogenesis of the latter²⁶.

Polymorphonuclear granulocytes (PMNs), and more specifically neutrophilic granulocytes, are the first-line of defense against infections and since they do not show any specificity for antigens, their predominant roles are cell killing and phagocytosis²⁷. Furthermore, PMNs are known to infiltrate tumors and constitute an important part of the immune infiltrate. They may be considered as potential antitumor cells, but clinical studies have been contradictory with respect to the function of tumor infiltrated PMNs. Whereas in patients with adenocarcinoma the presence of increased numbers of tumor infiltrating neutrophils was linked to poorer outcome²⁸, studies of gastric carcinoma suggested a correlation of neutrophil infiltration with good prognosis²⁹. In order to improve PMN recruitment for immunotherapy, bispecific antibodies (with one specificity for FcγRI and another for a tumor associated antigen) can be used. Results from a phase I clinical trial with a bispecific anti-FcγRI / anti-HER2/neu show that although the clinical course of the disease was not altered by the treatment, immunohistochemical analyses of tumor biopsies in individual patients documented infiltration of monocytes and PMN after antibody infusion³⁰. Interestingly, PMNs can be induced to produce substances that either stimulate or inhibit the angiogenic process. Activated PMNs contain granules which harbor matrix metalloproteinases capable of degrading the extracellular matrix^{27,31} and, hence, stimulate angiogenesis. In addition, they have been reported to produce a number of other angiogenic factors (VEGF, IL-8). On the contrary, PMNs are also known to be involved in the generation of endogenous angiogenesis inhibitors (e.g., angiostatin and other angiostatic factors (IL12, inducible protein 10 (IP10), interferon-γ (IFNγ))⁷. Despite this body of information, the actual role of infiltrated PMNs in tumors is poorly investigated and remains unclear. Next to these angiogenesis-related effects, PMNs are strong producers of chemotactic proteins to recruit other PMNs, monocytes, immature dendritic cells and T-lymphocyte subsets, in a self-sustained process^{7,27}.

Like PMNs, natural killer (NK) cells can recognize and lyse tumor cells without having to be primed and without the need for MHC recognition, and they are thought to be involved in immune surveillance against cancer^{7,21,32}. Although not frequently seen in tumors^{33,34}, there is some evidence that NK cells can play a critical role in angiogenesis inhibition by IL-12 induced IFNγ secretion³⁵. In a study by Yao et al³⁵ IL-12 was inoculated in a human Burkitt tumor in mice; subsequently, extensive vascular damage and tumor tissue necrosis was observed as well as an accumulation of activated NK cells in necrotic areas surrounding blood vessels. In another study by Hayakawa and co-workers³⁶ it was shown that IFNγ secreted by NK cells is involved in inhibition of tumor angiogenesis by alpha-galactosylceramide (α-GalCer), as presented by the MHC class-I like molecule

CD1d, a ligand for NK cells. Depletion of NK cell activity markedly reduced angiogenesis inhibition³⁵ and enhanced tumor growth³⁶, providing evidence that NK cells can play a critical role in the regulation of angiogenesis. Activated NK cells have also been reported to effectively kill both tumor cells and endothelial cell targets and, hence, inhibit angiogenesis^{37,38}. Therefore, NK cells can be considered to have a central role in inhibiting tumor angiogenesis at the interplay between host immunity and tumor-cell biology.

T-lymphocytes secrete a repertoire of molecules (e.g. IL-2, tumor necrosis factor- α (TNF α) and IFN γ) that regulate intercellular signalling events involved in host responses to foreign antigens, like recruitment of immune effector cells (IL-2, IFN γ) and regulation of expression of cell surface proteins on endothelial cells (TNF α)³⁹. T-cell infiltration is commonly seen in tumors (60% of an infiltrate can be composed of lymphocytes of which a large majority is T-cells³⁴) and is often associated with improved prognosis and survival^{33,40-42}. IFN γ secretion by CD4+ T cells of the Th1 subset has been shown to be important *in vivo* in inhibiting tumor cell proliferation, activating macrophages and enhancing the secretion of angiostatic chemokines⁴³. Interleukin-4 secretion by Th2 cells might be involved in tumor development as well by inhibiting both VEGF and bFGF induced angiogenesis *in vitro*⁴⁴. Although tumor infiltrating lymphocytes have been reported to show cytolytic activity induced by tumor cells³⁹, tumors have evolved mechanisms to escape their effects *in vivo*. Some tumors contain anergic lymphocytes, probably due to local secretion of immunosuppressive factors (i.e. TGF β ,⁴⁵) in the tumor microenvironment^{39,46} or the loss of MHC expression by tumor cells⁷. Alternatively, a decreased activity of the T-cells may be due to the presence of CD25-positive regulatory T-cells antagonizing a specific T-cell response. For this reason, current clinical immunotherapy approaches are combined with simultaneous infusion with antiCD25-antibodies to eliminate these regulatory T-cells^{47,48}. Although T-cell infiltrates in tumors are mostly seen as evidence of a protective immune response and, hence, associated with better prognosis, it has been demonstrated that both CD4+ and CD8+ lymphocytes are capable of promoting angiogenesis *in vivo* as well³⁹. Both in and outside a tumor T-lymphocytes can produce the angiogenic compounds bFGF and VEGF in response to hypoxia, suggesting an important role for these cells in tumor proliferation and metastasis^{39,49,50}. Although B-lymphocytes^{51,52} zet deze refs er maar achter are also described to be present in a tumor immune infiltrate, no specific information on angiogenesis-related effects of these cells has been found. However, since B-lymphocytes are a known source of cytokines like IL-1 and IL-6, some positive effects of this lymphocyte subset on the process of angiogenesis may be speculated.

Dendritic cells (DCs) are not often found in tumor infiltrates^{33,34} and if found, they are mostly in an immature state and incapable of inducing an effective immune response^{53,54}. Normally, DCs are the most efficient antigen-presenting cells with the unique ability to present tumor-specific antigens and subsequently activate a specific antitumor T-cell response *in vivo*. Immature DCs are capable of antigen ingestion and processing, but they must mature to activated DCs to elicit a productive immune response. Conflicting data have been published on the effect of infiltrated DCs on tumor development. Immature DCs have been shown to silence immunity and induce tolerance, either by deleting T-cells or by expanding regulatory T-cells^{53,55}. This phenomenon appeared to be mediated by tumor derived factors like VEGF, IL-6 and macrophage colony stimulating factor (M-CSF or CSF-1)^{23,54}. On the other hand, it was also shown that DC infiltration in tumor lesions is associated with improved survival rates and reduced incidence of recurrent disease in different types of malignancies⁵⁶. Additionally, immunotherapy using DCs has thoroughly been investigated and has proven to be feasible, nontoxic and effective in some patients, particularly when DCs are mature and active before administration^{53,57}. It is unknown, however, whether DCs themselves serve as angiogenesis mediators.

Mast cells are bone marrow-derived accessory cells which are not found in circulation (and do not migrate into tumor areas), but associated with certain structures as blood vessels or nerves⁶⁰. They are often associated with allergic reactions to certain allergens, but are also known to stimulate immunity by enhancing the inflammatory reaction. Mast cells have been found in a variety of human cancers (such as melanoma, breast and colorectal cancer) and their accumulation at tumor sites is associated with enhanced tumor growth and invasion^{11,60}. They have been reported to support tumor proliferation and progression by their ability to induce tumor angiogenesis⁶⁰. Activated mast cells are known to produce a number of angiogenic factors, like VEGF, bFGF, IL-8 and TNF α , and matrix metalloproteinase-9 (MMP-9, which is involved in degrading the extracellular matrix)^{11,60}. Furthermore, they are able to produce the angiogenic mediators histamine and heparin, which can stimulate endothelial cell proliferation (directly or indirectly by the stabilization of growth factors) and may have a role in the hyperpermeable nature of newly formed vessels in tumor angiogenesis⁶¹. Reduction of tumor mast cell density by transfection of a vector containing antisense stem cell factor cDNA (SCF, which stimulates mast cell migration, proliferation and degranulation) has been shown to inhibit angiogenesis and, therefore, tumor growth⁶². Therefore, interfering with mast cell recruitment to tumor sites might be a possible therapy for cancer.

When monocytes are released from the bone marrow into the blood, they are still immature. They only differentiate into macrophages once they are infiltrated into tissues⁶³. The majority of malignant tumors appears to be infiltrated by macrophages, which can comprise up to 80% of the total tumor mass^{33,64,65}. These tumor associated macrophages (TAMs) are capable of killing tumor cells and releasing angiostatic compounds, but they can also stimulate tumor growth by producing angiogenic factors and metalloproteinases^{7,65}. During tumor development the tumor stimulating effects of these infiltrated macrophages often overrule the tumor inhibiting ones⁷. Overexpression of macrophage chemoattractants within tumors has been shown to correlate with poor prognosis⁶⁶ and the level of macrophage infiltration in tumors is known to be positively correlated with microvessel density, tumor stage and angiogenesis in tumors⁶⁷. TAM presence is, therefore, associated with reduced relapse-free and overall survival^{65,67-70} in several tumor types (i.e. melanoma⁶⁷, cervical^{68,69} and breast^{65,70} cancer). On the other hand, however, in other tumor types (i.e. prostate⁷¹ and stomach⁷² tumors) TAM presence appears to be associated with improved prognosis. It is suggested that extensive monocyte invasion in a tumor results in elimination of tumor mass, while a low level of monocyte recruitment provides an angiogenic stimulus which permits tumor proliferation⁷³.

All together, a tumor infiltrate both stimulate and inhibit angiogenesis, depending on the type, timing, number and activation state of all the cells present⁷. In the next part of this review, we will focus more explicitly on the recruitment and the possible angiogenic and angiostatic activities of tumor-associated macrophages (TAMs).

RECRUITMENT OF TAMs BY TUMORS

Chemokines and cytokines

Monocytes, like other leukocytes, are actively attracted to the tumor site due to the production of cytokines and chemokines by tumor cells. Chemokines are divided in major subfamilies, like CXC and CC, depending on the arrangement of the 2 N-terminal cysteine residues. They play major roles in all aspects of tumor biology, including recruitment of leukocytes, regulation of angiogenesis and tumor growth (mainly CXC chemokines⁷⁴), and control of the movement of tumor cells during

metastasis⁷⁵. Several CC chemokines, like monocyte chemotactic protein-1 (MCP-1 or CCL2) and RANTES (or CCL5), produced by tumor cells, endothelial cells, fibroblasts and also by TAMs, are known to attract monocytes^{75,76}. MCP-1 is highly expressed in several tumor types and is associated with infiltration of monocytes. Interestingly, MCP-1 expression has been shown to correlate significantly with levels of VEGF, TNF α and IL-8, suggesting a possible role for MCP-1 as an angiogenesis regulator as well^{63,77,78}. The expression of another CC chemokine, RANTES, in breast tumor cells is known to be elevated synergistically by IFN γ and TNF α , regulating monocyte migration into tumor sites and promoting cancer progression. Moreover, RANTES has been found to stimulate monocytes to secrete MMP-9 and MMP-19, indicating its indirect involvement in degrading the basement membrane and, therefore, in angiogenesis^{79,80}. Although the involvement of CXC chemokines in regulating angiogenesis is well studied⁷⁴, their role in directing tumor infiltration by TAMs is less well defined. The angiogenic factor IL-8 (CXCL8), however, has been shown to cause rolling monocytes to adhere firmly onto endothelial cell monolayers expressing E-selectin, indicating that IL-8 is not only acting as a chemoattractant but is also involved in translating initial monocyte tethering into firm adhesion through activation of leukocyte integrin⁸¹.

Monocytes are directed to the tumor by other cytokines as well. While VEGF is a well known angiogenic cytokine, it is also a chemotactic protein for monocytes via activation of flt-1 (vascular endothelial growth factor receptor-1)^{63,82-84}. It is produced by both tumor cells and TAMs and is controlled by oxygen tension⁸⁵. Since VEGF is mainly expressed in response to hypoxia, TAMs are often seen to accumulate in poorly vascularized tumor areas⁶³. Macrophage colony stimulating factor (M-CSF or CSF-1) is another cytokine with chemoattractive properties for monocytes and it is being produced by tumor cells and TAMs. Elevated expression of M-CSF and its receptor (CSF-1R) have been found in human breast, endometrial and ovarian tumors^{86,87}. High M-CSF expression is associated with high TAMs accumulation in breast carcinomas⁸⁸ and poor prognosis⁸⁹⁻⁹¹. It has also been described to be involved in induction of VEGF and IL-8, suggesting a possible role in regulation of angiogenesis for this cytokine as well^{77,92}.

Adhesion molecules

Next to chemoattractants, adhesion molecule expression on endothelial cells as well as on monocytes are eminently involved in monocyte recruitment to tumors^{19,20,93}. To actually infiltrate tissues, monocytes have to interact with the venular vessel wall. This process of monocyte-vessel wall interaction appears to be a multistep cascade in which each step has to be completed for the next step to occur⁹⁴. The first step is the tethering and, subsequently, rolling of leukocytes along the vessel wall. Monocyte rolling is mainly mediated by adhesion molecules of the selectin family (L-selectin found on monocytes and both P- and E-selectin on activated endothelial cells), binding to their counterreceptors on opposing cells (e.g. CD34 on endothelial cells and P-selectin glycoprotein-1 (PSGL-1) on monocytes)⁹⁵. Rolling monocytes can become activated by chemoattractants or cell contact-mediated signals inducing secondary adhesion molecules⁹⁶, by which they can firmly adhere to the endothelium. Monocyte adhesion to the vessel wall is mediated by adhesion molecules of two families: the integrins (e.g. LFA-1 and VLA-4, expressed on monocytes) and the immunoglobulin gene superfamily (e.g. ICAM-1 and VCAM-1, expressed on activated endothelial cells and on leukocytes or macrophages)⁹⁷. Adhering monocytes can then transmigrate through intercellular junctions between endothelial cells and migrate into the surrounding (tumor) tissue⁹⁸.

Previously we have shown that tumor-derived angiogenic factors such as VEGF and bFGF are responsible for downregulation of endothelial adhesion molecules such as ICAM-1, VCAM-1 and E-selectin, resulting in diminished leukocyte-vessel wall interactions in tumor vessels^{18,99,100}.

ANGIOSTATIC PROPERTIES OF TUMOR ASSOCIATED MACROPHAGES.

Macrophages are known to accumulate mainly in poorly vascularized, hypoxic areas, due to specific upregulation of various chemoattractants (recently reviewed by Murdoch et al⁷⁵). Once macrophages arrive at the tumor site they start to produce their own set of proteins to attract more leukocytes and to influence the process of angiogenesis. TAMs are reported to have a number of angiostatic properties (Table 5.1), which will be discussed in this section.

Table 5.1: Angiostatic factors secreted by TAMs

Cytokines/chemokines	Regulation and effects	References
IL-12	-IL-12 → ↑ IFN γ and TNF α by Th1 cells → ↑ angiostatic factors IP10 (CXCL10) and MIG (CXCL9)	63,101,103-107,109
IL-18	-IL-18 → ↑ IFN γ by Th1 cells → ↑ angiostatic factors IP10 (CXCL10) and MIG (CXCL9)	101,104,108,114-117
MMP-12	-↓ bFGF-induced endothelial cell proliferation -↓ M-CSF/IL-1/TNF α /VEGF → ↑ MMP-12 → ↑ cleavage of plasminogen to angiostatin	27,63,118,119,121,122

Interleukin-12

Interleukin-12 (IL-12) is produced by monocytes/macrophages and also by dendritic cells and B-cells; it is known for its antitumor and antimetastatic activity¹⁰¹. IL-12 activates a T-cell-dependent antitumor immune response that is able to eradicate established large tumors in a number of immunogenic tumor models¹⁰². Moreover, activated macrophages appear to function as effector cells in this IL-12-induced tumor eradication by inducing apoptosis¹⁰². IL-12 appears to have angiostatic capacities as well^{103,104}, which induce tumor necrosis¹⁰⁵. Angiogenesis inhibition by IL-12 is not carried out via direct interaction with endothelial cells, but merely via initiation of IFN γ production by Th1 cells and NK cells^{63,106,107}. The production of IL-12 by macrophages enhances immune function by shifting CD4+ cells towards the Th1 subset, which secrete IL-2 and IFN γ ¹⁰⁸, facilitating in turn the proliferation and activation of CD8+ cytotoxic T cells, NK cells and macrophages, which may contribute to tumor regression¹⁰⁴. IFN γ is, in turn, able to induce the production of IFN-inducible protein 10 (IP10 or CXCL10) and monokine induced by IFN γ (MI or CXCL9), which both have angiostatic properties^{105,109}. Moreover, IFN γ is able to induce additional non-antigen specific antitumor mechanisms, including a retardation of cellular proliferation and production of nitric oxide¹⁰⁴. In a murine model of breast cancer, IL-12 therapy has shown potent angiostatic effects and subsequent tumor regression. Levels of tumor VEGF and MMP-9 were declined markedly and eventually undetectable¹¹⁰. IL-12 is able to accelerate a variety of anti-tumor mechanisms, some of which are antigen specific and some of which are not. At the moment, the use of IL-12 is investigated in phase I and II trials with promising results, including improved immunological antitumor responses¹¹¹⁻¹¹³.

Interleukin-18

IL-18 is another substance known to induce tumor regression via angiogenesis inhibition¹⁰⁴. It is being produced by (activated) macrophages, dendritic cells and Kupffer cells^{101,104}. Similar to IL-12, IL-18 inhibits angiogenesis through stimulation of IFN γ production (by both Th1 lymphocytes and

NK cells), resulting in a systemic antitumor response¹⁰⁸. It appears to be more potent as IFN γ inducer than IL-12, apparently through a separate intracellular signal-transduction pathway^{114,115}. IL-18 treated tumors show a significant decrease of microvessel density¹¹⁶. Administration of IL-18 inhibits the growth of fibrosarcoma by hypovascularization¹¹⁵. One of the angiostatic mechanisms of IL-18 is its ability to inhibit bFGF-induced endothelial cell proliferation which was demonstrated *in vitro*, while IL-12 lacks such an effect¹¹⁶. Therefore, the angiostatic pathway of IL-18 may not be completely dependent on activation of IFN γ . Interestingly, IL-18 and I-12 have been reported to have synergistic antitumor effects^{115,117} and synergistically inhibit angiogenesis¹⁰⁴. Systemic administration of IL-18 was shown to potentiate inhibition of proliferation of carcinoma cells modified to express IL-12¹¹⁵. In a murine sarcoma model, intratumoral injection of dendritic cells engineered to produce IL-12 or IL-18 showed to decrease tumor proliferation and even complete tumor rejection, more strongly than either one of them¹¹⁷. Beside its inhibitory effects on angiogenesis, IL-18 has also proven to be a proinflammatory cytokine and a strong inducer of immune responses¹¹⁵. Taken together, IL-18, particularly in combination with IL-12, may be a promising antitumor agent.

Matrix metalloproteinase-12

Matrix metalloproteinases (MMP) are a family of zinc-dependent endopeptidases which can cleave all major molecules of the extracellular matrix^{63,118,119}. During tumor growth, MMP can enhance angiogenesis and degrade connective tissue and basement membranes to enable tumor growth and metastasis¹¹⁸. Next to a tumor promoting effect, some MMP, like metalloelastase (MMP-12), matrilysin (MMP-7) and gelatinase-B (MMP-9) have also been shown to possess angiostatic properties^{118,119}. MMP-12 is generally expressed in macrophages, although tumor cells were shown to express this enzyme as well^{118,120}. Pro-MMP-12 is released by macrophages in response to several factors (like M-CSF, IL-1, TNF α and VEGF). The amount of macrophage-derived MMP-12 appears to be higher in grade I and II vulvar tumors compared to the more aggressive grade III tumors, indicating that the level of MMP-12 positive macrophages correlates with a better prognosis¹¹⁸. Moreover, in a study of colorectal carcinoma, patients with high expression of MMP-12 mRNA significantly demonstrated better survival compared with those patients who did not show high MMP-12 expression. Patients with high MMP-12 expression showed lower microvessel density (MVD), demonstrating that MMP-12 is able to inhibit angiogenesis *in vivo* in human tumors¹²¹. MMP-12 is capable of generating angiostatin¹²², a potent angiogenesis inhibitor identified by O'Reilly et al¹²³ in Lewis lung carcinoma. Angiostatin is a cleavage product of plasminogen, which has been shown to inhibit endothelial cell proliferation and impairs both primary and metastatic tumor growth through inhibition of angiogenesis¹¹⁹. All together, MMP-12 and angiostatin appear to play an important role in the inhibition of angiogenesis and tumor progression in patients.

ANGIOGENIC PROPERTIES OF TUMOR ASSOCIATED MACROPHAGES.

In contrast to the limited knowledge concerning angiostatic activities of TAMs, more research has focused on their angiogenic properties (Table 5.2). Understanding the angiogenic activity of TAMs in a tumor will hopefully rise opportunities to counteract these activities in order to treat cancer.

Interleukin-1

The cytokine interleukin-1 (IL-1) is produced mainly by monocytes/ macrophages and appears to be a highly inflammatory cytokine, also possessing various immune, degradative and growth

Table 5.2 : Angiogenic factors produced by TAMs

Cytokines/chemokines	Regulation and effects	References
	-IL-1, -IL-1, → ↑ HIF → ↑ VEGF -IL-1, → ↑ MIP-2/HGF/TNFα -IL-1, → ↑ angiogenin	63,124-127
VEGF	-Hypoxia → ↑ HIF → ↑ VEGF -M-CSF → ↑ VEGF -↑ TNFα/IL-1 → ↑ VEGF -Chemotactic for monocytes	92,130,140
IL-8	-Activation NFκB → ↑ IL-8 -↑ TNFα/IL-1 → ↑ IL-8 -M-CSF/MCP-1/TGF, → ↑ IL-8	77,78,135,140
bFGF	-↑ TNFα/IL-1 → ↑ bFGF -bFGF → ↑ VEGF -Chemotactic for monocytes	137,138,140
TNFα	-TNFα → ↑ TP → ↑ angiogenic factor deoxyribose 1-phosphate -TNFα → ↑ IL-8/VEGF/bFGF/angiogenin -TNFα → ↑ IL-8/VEGF/bFGF receptors	63,66,67,127
MMP-9	-↑ TNFα/LPS → ↑ MMP-9 -VEGFR-1 → ↑ MMP-9 -↑ MMP-9 → ↑ VEGF	118,143,144,162
M-CSF	-M-CSF → ↑ VEGF/IL-8 -Chemotactic for monocytes	77,92
MCP-1	-MCP-1 → ↑ VEGF/IL-8/TNFα -Chemotactic for monocytes	77,78
MIF	-MIF → ↑ TNFα/IL-1/CXC chemokines	148,149
uPa	-degradation extracellular matrix	63,66,147
PAF	-PAF → ↑ TNFα/IL-1/bFGF/VEGF	150
TGFB	-TGFB → ↑ IL-8/TNFα/VEGF/bFGF/IL-1 -Chemotactic for monocytes	78
HB-EGF	-HB-EGF → ↑ VEGF/MMP-9/MMP-3 -VEGF → ↑ HB-EGF	151
PDGF	-Macrophage recruitment and migration -Vessel stabilization	33,152

promoting properties. There are two IL-1 proteins, IL-1α and IL-1β, and one naturally occurring IL-1 receptor antagonist (IL-1Ra)¹²⁴. All IL-1 associated proteins bind to the same receptor, but exert different effects. Both IL-1α and IL-1β are involved in tumor angiogenesis, but the role of IL-1β appears to be more evident¹²⁴. This is probably due to the fact that IL-1β is secreted in the tumor microenvironment and, hence, activates tumor cells. IL-1α has a less prominent effect, since this cytokine is largely cell-associated and remains cytosolic¹²⁴. It is demonstrated by Song et al¹²⁵ that IL-1α potentiates the development of an antitumor immune response, thereby limiting tumor growth, whereas IL-1β enhances invasiveness of the tumor and induces angiogenesis and immune suppression in the patient. IL-1β has been shown to selectively induce the expression of HIF-1, thereby promoting

VEGF production. Macrophages used from IL-1 α or IL-1, knockout mice showed reduced VEGF secretion¹²⁴. It has been demonstrated that IL-1 β is able to accelerate tumor growth of Lewis lung carcinoma (LLC) in transplanted mice by neovascularization and induction of angiogenic factors, like VEGF, macrophage-inflammatory protein-2 and human growth factor¹²⁶. Another effect of IL-1 β on angiogenesis is through its involvement in the production of TNF α ¹²⁴ and angiogenin¹²⁷. The properties of IL-1 associated with tumor growth and metastasis make it an attractive target for therapeutic intervention. Bar et al¹²⁸ genetically engineered NIH/3T3 cells to continuously secrete IL-1 receptor antagonist and encapsulated these cells within microspheres to provide a barrier for the immune system. The microspheres were injected subcutaneously in mice with fibrosarcoma cells. It was shown that both tumor development and tumor angiogenesis were inhibited, and that survival was prolonged. In conclusion, as many tumors and TAMs have been shown to express IL-1, IL-1 receptor antagonist may be useful in anti-cancer therapies.

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) promotes endothelial cell proliferation and new blood vessel formation, and is produced by TAMs and also by tumor cells in a variety of human cancers⁶³. Although there are multiple angiogenic factors that promote blood vessel formation in human cancer, VEGF appears to play a dominant role⁹². The VEGF family consists of the prototype member VEGF (also referred to as VEGF-A), placenta growth factor (PlGF), VEGF-B, VEGF-C and VEGF-D. Besides VEGF, TAMs also express VEGF-C and VEGF-D, both associated with the formation of lymphatic vessels and lymphatic metastasis¹²⁹. Next to a stimulatory effect on the process of angiogenesis, VEGF increases vascular permeability¹³⁰ and prevents the upregulation of endothelial adhesion molecules induced by inflammatory cytokines¹⁸. At present, two VEGF receptors, VEGFR1 (or Flt-1) and VEGFR2 (or Flk-1) are known. While inactivation of both receptors resulted in embryonic lethality, there is now consensus that VEGFR2 is mainly involved in the angiogenic, mitogenic and permeability enhancing effects of VEGF¹³⁰, while VEGFR1 is likely to have important roles in the recruitment of monocytes to tumors^{82,83} and in the induction of MMP-9¹³¹. VEGF expression in macrophages¹³² and tumors is induced via hypoxia-inducible factor (HIF), a major mediator of hypoxic responses, regulated by the product of von Hippel-Lindau (VHL) tumor-suppressor gene (for review see Mole et al¹³³). In monocytes, VEGF is also induced via M-CSF and MCP-1, indicating a role for these macrophage chemoattractants in angiogenesis regulation^{77,92}. TAMs preferentially migrate to hypoxic areas within tumors and strongly express HIF-2 α , while normal tissue macrophages do not, resulting in expression of HIF-2 α responsive angiogenic factors, like VEGF, and high tumor vascularity. This high macrophage HIF-2 α appears to be an independent prognostic factor for poor outcome. The mechanisms up-regulating HIF-2 α in macrophages may include inflammatory cytokines as well as hypoxia¹³⁴.

Tumor growth inhibition has been demonstrated by several laboratories using a number of anti-VEGF approaches, including anti-VEGFR-2 antibodies¹³⁰. *In vivo* treatment of mice with anti-VEGF antibodies was shown to result in higher leukocyte-vessel wall interactions in tumor vessels compared to non-treated animals¹⁸. Moreover, combining anti-VEGF treatment with chemotherapy or radiation results in greater antitumor effect than either treatment alone, indicating that anti-VEGF approaches for the treatment of cancer are promising.

Interleukin-8

Interleukin-8 (IL-8) is a potent angiogenic factor in several cancers and associated with metastasis¹³⁵. It has been reported to be produced by TAMs and by endothelial cells, fibroblasts

and tumor cells¹³⁵. Monocytes can be induced to upregulate IL-8 secretion by M-CSF, MCP-1 and TGF β , suggesting an important role for these chemotactic factors in monocyte/macrophage-mediated tumor angiogenesis^{77,78}. The interaction between infiltrated macrophages and tumor cells is also known to upregulate IL-8 (and VEGF) expression in both cell types, in a paracrine fashion^{78,135}. TAMs produce TNF α and IL-1, which in turn induce tumor cells to produce higher levels of IL8 and VEGF⁷⁸. IL-8 expression has been correlated negatively with patient survival and positively with macrophage infiltration in tumors, suggesting that IL-8 might be dominantly derived from TAMs⁶⁸. In contrast to these results, Chen et al.¹³⁵ reported that cancer cells were the major source of IL-8. There is a significant positive correlation between IL-8 expression and microvessel density¹³⁶, indicating the angiogenic potential of this interleukin. The induction of IL-8 mRNA expression involves activation of the NF κ B pathway, which can be suppressed >93% by anti-inflammatory agents¹³⁵. Specific inhibition of tumor angiogenesis via NF κ B and IL-8 using anti-inflammatory agents, like aspirin, might be a new approach to inhibit angiogenesis in cancer¹³⁵.

Basic fibroblast growth factor

Basic fibroblast growth factor (bFGF or FGF-2) is secreted by macrophages, mast cells, lymphocytes, fibroblasts and by some malignant cells^{64,137,138}. bFGF belongs to a large family of homologous polypeptide growth factors and promotes every phase of the angiogenic process, including synthesis of proteinases, endothelial cell migration and differentiated capillary tube formation *in vitro*¹³⁷. bFGF induces angiogenesis through the high-affinity heparin binding receptors FGFR-1 and FGFR-2⁷⁶. The number of bFGF positive mast cells and macrophages appears to be higher in pancreatic adenocarcinomas with bFGF positive cancer cells¹³⁸, suggesting a cohesion between bFGF expression in these cell types. In various human tumors bFGF has also been localized to stromal macrophages and *in vitro* experiments showed expression of bFGF in macrophages, induced by tumor derived signals⁶⁴. Increased bFGF expression correlates with poor prognosis and bFGF expression by cancer cells shows a direct correlation with microvessel density¹³⁸. Stromal bFGF expression, however, did not correlate with either microvessel density or the frequency of metastasis in pancreatic carcinoma¹³⁷. bFGF has been found to be chemotactic for macrophages¹³⁸, implying that this factor also has indirect stimulatory effects on angiogenesis and negatively affects patient survival.

Another way for bFGF to indirectly induce angiogenesis is by its capacity to promote VEGF synthesis. In addition, there is a synergistic interaction between bFGF and VEGF in inducing endothelial cell proliferation and angiogenesis^{76,137}. Zhang and Issekutz⁷⁶ reported that bFGF significantly reduces monocyte adhesion and migration to both resting and stimulated endothelial cells, even when these monocytes were activated with chemotactic factors like RANTES or MCP-1⁷⁶. The underlying mechanism appeared to be bFGF-induced downregulation of the endothelial adhesion molecules ICAM-1, VCAM-1 and E-selectin, that are necessary for leukocyte capture, rolling, firm adhesion and emigration^{95,139}. This effect of bFGF might interfere with infiltration of monocytes and other leukocyte subtypes in tumors. Overall, TAM-derived bFGF seems to be a less prominent angiogenic factor as compared with the other factors described in this review. Since its expression has been correlated with decreased patient survival, bFGF presumably exerts its tumor growth stimulating effect via ways other than direct promotion of angiogenesis.

Tumor necrosis factor- α

Tumor necrosis factor- α (TNF α) is an inflammatory cytokine with angiogenic activity that is mainly expressed by TAMs⁶⁶. Other sources are mast cells, T-lymphocytes, NK cells, neutrophils and some

tumor cells¹⁴⁰. This cytokine has been found to promote the production of angiogenic factors, like IL-8, bFGF and VEGF, through activation of transcription factors, in both tumor cells and surrounding other cell types^{67,140}. The *in vitro* tube formation in vascular endothelial cells that is induced by TNF α , is inhibited by administering anti-IL-8, anti-VEGF and anti-bFGF antibodies. When all these antibodies were administered together, an almost complete abrogation of tube formation was seen. *in vivo*, anti-IL-8 and anti-VEGF antibodies were shown to block angiogenesis induced by TNF α in rabbits cornea¹⁴⁰. TNF α also stimulates tumor cells to express angiogenin, a potent angiogenic cytokine¹²⁷. Angiogenin has found to be up-regulated in pancreatic cancer patients and contributed to the aggressiveness of pancreatic cancer. In colorectal cancer, increased serum angiogenin concentration is correlated with cancer progression¹²⁷. TNF α is also known to upregulate the expression of the angiogenic enzyme thymidine phosphorylase (TP) in cancer cells⁶⁶. TAM expression of TNF α has been correlated positively to upregulation of TP in breast tumors¹⁴¹. TP catalyses the reversible conversion of thymidine to thymine and the production of the angiogenic factor deoxyribose 1-phosphate, thereby influencing the angiogenic process. TP expression in breast TAMs appears to correlate with increased microvessel density and poor prognosis in patients with various tumor types⁶⁶.

TNF α also up-regulates the expression of endothelial cell adhesion molecules, a necessary step for leukocyte-vessel wall interactions and, hence, leukocyte infiltration into the tumor to occur. Previously we have shown¹⁸ that TNF α induces a decrease in the number of circulating leukocytes in nude mice bearing a small LS174T colon carcinoma in their ear. This is probably due to the fact that TNF α induces leukocyte adhesion systemically. This effect was evident for both mononuclear cells (MMNs) and polymorphonuclear cells (PMNs), but was most prominent for MMNs (80% reduction versus 55% for PMNs ($p < 0.01$)). In contrast to a small ear tumor, the presence of a large flank tumor tended to mask these TNF α induced changes, suggesting a systemic effect of tumor-derived signals on endothelial cells adhesion molecule expression and, hence, leukocyte-vessel wall interactions. In summary, TNF α is seen as a potent activator of tumor angiogenesis, which is modulated by various angiogenic factors, and an important factor in recruiting leukocytes to a site of injury or a tumor.

Matrix metalloproteinase-9

Expression of matrix metalloproteinase-9 (MMP-9), or gelatinase-B, is directly associated with increased tumor invasiveness and metastasis as well as poor patient outcome^{120,142}. MMP-9 is expressed by cancer cells, stromal neutrophils, fibroblasts, mast cells and macrophages¹¹⁸. In a variety of invasive cancers, TAMs have been shown to be a major source of MMP-9⁶³. MMP-9 expression appears to be significantly higher in stage III and IV neuroblastoma than in stage I or II, suggesting an important role for this proteinase in tumor progression. Stromally derived MMP-9 has recently been shown to contribute to angiogenesis by promoting blood vessel morphogenesis and pericyte recruitment and, hence, vessel stabilization¹⁴³. In monocytes/macrophages, MMP-9 is induced by inflammatory mediators like TNF α and bacterial lipopolysaccharide (LPS)¹⁴⁴. VEGFR-1 is also described to be involved in MMP-9 induction in macrophages¹⁴⁵, while MMP-9, in turn, increases availability of VEGF¹⁴³. Furthermore, leukocyte infiltration and subsequent activation of MMP-9 expression has been associated with the 'angiogenic switch' in premalignant tissue and it promotes tumor angiogenesis in mouse models¹¹⁸. MMP-9 is known to degrade the vascular basement membrane by cleavage of type IV collagen¹⁴⁴. Despite the angiogenic effects of this matrix metalloproteinase, MMP-9 has also been described to generate angiostatin, implying its involvement in angiogenesis inhibition as well¹¹⁸. The net result, however, of MMP-9 presence in tumors seems

to be promotion of angiogenesis. Further evidence that MMP-9 contributes to tumor progression came from studies on their endogenous tissue inhibitors (TIMPs). Overexpression or intraperitoneal injection of TIMPs was shown to reduce metastasis in experimental models¹⁴⁶. However, effects of synthetic metalloproteinase inhibitors in phase III clinical trials have been disappointing, showing no significant difference in patient survival¹⁴⁶. It remains to be seen whether these inhibitory substances become a standard cancer treatment.

Other angiogenic factors produced by TAMs

Beside the earlier mentioned angiogenic substances, TAM also express a number of other angiogenic factors (Table 5.2). Among them are the chemotactic proteins M-CSF and MCP-1, which induce both VEGF and IL-8; they have already been described previously in this manuscript. Furthermore, TAMs express urokinase-type plasminogen activator (uPA) and its receptor uPAR (CD87)^{63,66,147}, macrophage inhibitory factor (MIF)^{148,149}, platelet activating factor (PAF)¹⁵⁰, transforming growth factor- β (TG- β)⁷⁸, heparin-binding epidermal growth factor (HB-EGF)¹⁵¹, and platelet derived growth factor (PDGF)^{33,152}, which are described further in Table 5.2. Although these angiogenic factors might be of equal importance, knowledge about them is limited compared with the ones described in this review.

TUMOR ASSOCIATED MACROPHAGES (TAMs) IN IMMUNOTHERAPY?

The data presented here on angiogenic and angiostatic activities of TAMs clearly show two faces of the presence of these cells in tumors. It is possible that tumors are eradicated by TAMs in a preclinical stage of cancer development. In most tumors studied, however, the balance between angiogenic and angiostatic effects of TAMs is in favor of the angiogenic ones. The ability of TAMs to infiltrate tumor tissue and their presence at the tumor site can and have been used to counteract their stimulating activities via immunotherapy, as described in the following section.

Although macrophages have the potential to mediate tumor cytotoxicity and to stimulate antitumor lymphocytes, some tumors are -apparently- capable of escaping these defence mechanisms. This could be due to the fact that malignant cells often do not express 'foreign' surface antigens, and are not recognized as such by circulating monocytes. It has also been suggested that tumor-derived signals suppress TAM activity, because neither monocytes isolated from peripheral blood of cancer patients nor TAMs obtained from tumor sites showed any cytotoxicity towards tumor cells *ex vivo*. Moreover, macrophages isolated from metastatic tumors were shown to suppress tumor specific T and NK cytotoxicity^{63,108}. Even worse, tumors seem to benefit from the angiogenic capacities of TAMs, as described in this review. Nevertheless, the ability of macrophages to infiltrate tumors can be used to develop immunotherapeutic approaches for the treatment of cancer. In addition, it may be possible to emphasize their angiostatic properties via gene therapy.

Adoptive transfer of *ex vivo* generated macrophages in patients with cancer may be a way of overcoming immune silencing effects of the tumor micro-environment. This was performed for the first time by Fidler and co-workers in 1974¹⁵³, using the B16 melanoma model. They demonstrated suppression of pulmonary metastases following infusion of *ex vivo* activated macrophages. This observation was confirmed by the results of additional rodent studies. However, regression of the primary tumor was seldom seen¹⁵⁴. In humans, monocytes have been isolated from the blood of cancer patients, matured (using M-CSF), activated (using LPS or IFN γ) and, subsequently, infused back into the patient. Apart from low grade fever and other flu-like symptoms, no side effects were observed. There was, however, no clinical evidence of an antitumor effect of these *ex vivo* activated

macrophages¹⁵⁴. This may be explained by the observation that the infused macrophages concentrated only partially at sites of metastases, while many cells became trapped in lungs, spleen, liver or kidneys, being unable to exert a proper antitumor effect^{75,154}. This may be due to the fact that macrophages instead of monocytes were used in these studies, because monocytes are the cells that are normally recruited from the vasculature into a tumor. The cytokines and chemokines released by tumors may be less effective in recruiting macrophages than their precursor cells⁷⁵.

Macrophages have been transfected *ex vivo* with anti-cancer genes such as IFN γ and M-CSF¹⁵⁵, or therapeutic genes¹⁵⁶; they may also be tagged with chemotherapeutic or angiostatic drugs. Griffiths et al.¹⁵⁶ used an adenoviral vector to transfect human macrophages with a hypoxia response element (HRE)-regulated p450 gene construct. The p450 enzyme converts the pro-drug cyclophosphamide to an active toxic form, which is also known to possess angiostatic properties¹⁵⁷. Transfected macrophages migrated into the hypoxic centre of tumor spheroids. When cyclophosphamide was subsequently administered, significant tumor killing was seen within the tumor mass. It is not yet known, however, whether p450 expression in healthy, normoxic tissues is low enough to avoid killing cells in these areas. Nevertheless, targeting hypoxic tumor tissue may prove to be a successful innovative form of gene therapy strategy.

Another way to perform gene therapy is to use macrophage function itself as an *in vivo* target. Except for secreting a variety of angiogenic cytokines and enzymes, macrophages are able to exert tumoricidal functions after stimulation with IFN γ and LPS, like phagocytosis of apoptotic tumor cells and presenting tumor-associated antigens to T-cells¹⁵⁸. By transfecting macrophages with genes encoding for tumor antigen specific monoclonal antibodies, they become able to specifically kill tumor cells which express the antigens *in vitro*¹⁵⁹. Whether this approach will be as effective *in vivo* remains to be seen.

Despite the fact that non-specific immunotherapy with cytokines has resulted in some dramatic antitumor responses, it has not been as effective as anticipated¹⁶⁰. When using non-specific forms of immunotherapy one has to keep in mind that the recruitment of some leukocyte subsets might be detrimental for patient survival. On the other hand, failure of specific (T cell mediated) immunotherapy might be explained by the fact that immune cells are unable to infiltrate the tumor in quantities large enough to effectively destroy it. Since endothelial cells are involved in the selection of leukocyte subsets to infiltrate a tumor, tumors have used this endothelial cell property to develop a mechanism to escape immune infiltration. We and others have previously shown that tumor endothelial cells have a suppressed expression of adhesion molecules^{18,21,100}, thereby reducing leukocyte-vessel wall interactions. This downregulation has been shown to be due to exposure to tumor-derived angiogenic factors like bFGF and VEGF^{18,21,99}. Recently, we have demonstrated¹⁶¹ that angiogenesis inhibition can be a way to increase leukocyte-vessel wall interactions in tumors, making them more vulnerable to various immunotherapeutic approaches. However, angiostatic therapy will not result in the attraction of specific leukocyte subsets, such as cytotoxic T-lymphocytes, which may be able to destroy the tumor. Macrophages are clearly not the type of immune cells desired in a tumor, because of their tumor supporting capabilities. However, since macrophages have been shown to produce a variety of angiogenic factors, it is expected that anti-angiogenesis therapy will also target and suppress macrophage pathways involving angiogenesis. Moreover, as monocytes/macrophages are known to infiltrate tumors, these cells may be exploited as vehicles or delivery systems for angiostatic compounds to perform anti-angiogenic treatment *in situ* and possibly avoid negative side effects of this treatment strategy on other physiological processes associated with angiogenesis.

CONCLUSIONS

Tumor associated macrophages are important regulators of angiogenesis and, hence, play a major role in tumor progression and metastasis. A high macrophage density has been associated with poor prognosis for cancer patients and with failure of immune functions, indicating a role for macrophages in assisting the tumor to escape from immune surveillance. Macrophages have been used for immunotherapeutic and gene therapeutic treatments, but until now the results obtained here were not satisfactory. Another line of research has shown that in tumors leukocyte-vessel wall interactions are suppressed, resulting in reduced recruitment of inflammatory cells that can be detrimental to the tumor, like cytotoxic T-cells and dendritic cells. Angiostatic therapy has been shown to upregulate leukocyte-vessel wall interactions in tumors and, hence, the recruitment of immune cells that can destroy a tumor. We hypothesize that combining immunotherapy with angiostatic therapy will result in improved, and possibly synergistic, anti-tumor responses and better prognosis for cancer patients.

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CHAPTER 6

General Discussion

GENERAL DISCUSSION

In European and American countries, cancer is one of the most important causes of death and despite persisting efforts, progress in improving cancer therapy has been modest. Cancer cells are heterogeneous in origin and are genetically unstable, which makes it difficult to develop anti-cancer strategies¹. Angiogenesis, the formation of new blood vessels out of pre-existing ones, plays a prominent role in tumor development. In 1971, Folkman and colleagues proposed that, because tumor growth and metastasis are dependent on angiogenesis, blocking angiogenesis could be a strategy to arrest tumor proliferation². In addition to its effect on tumor development, the process of tumor angiogenesis appears to have an inhibiting effect on the function of the immune system^{3,7}. Therefore, inhibition of tumor angiogenesis may result in tumor starvation as well as an improved anti-tumor immune response. For the development of improved anti-cancer strategies and cancer treatment it is necessary to understand the process of angiogenesis and its effects on the immune system.

To develop anti-angiogenesis based cancer therapies, various *in vitro* and *in vivo* assays are being used to investigate blood vessel formation and leukocyte-vessel wall interactions. These assays all have their own advantages and limitations. Generally, *in vitro* tests (like cell proliferation assays, migration assays and flow chamber experiments) are cheaper, faster and have fewer variables to be controlled by the researcher. However, the major disadvantage of such *in vitro* experiments is that the data obtained cannot be extrapolated to the *in vivo* situation straight away. When carried out carefully, *in vivo* assays generally provide more accurate information on the processes studied; they are the best and only basis for pre-clinical drug testing. *In vivo* angiogenesis assays include microcirculatory tissue preparations (like the dorsal skin fold chamber⁸, skin flaps⁹ and the chick chorio allantoic membrane (CAM) assay¹⁰) and implantations of biocompatible polymer matrix (like the sponge models¹¹ and Matrigel-plug assays¹²), in which compounds of interest can be directly injected. The matrix implantation assays can be used to rapidly screen factors that either stimulate or inhibit angiogenesis. They are, however, expensive and animals have to be sacrificed before analysis can take place¹¹. Only microcirculatory tissue preparations can be used to study leukocyte-vessel wall interactions in angiogenic vessels. These animal models are relatively time consuming, carry a high risk of failure and may induce an inflammatory reaction by surgical procedures at the site of interest. In the present study we have developed a new and simple mouse model in which both angiogenesis and leukocyte-vessel wall interactions in and outside a tumor can be quantified non-invasively, allowing longitudinal analysis during tumor development and/or in the course of anticancer treatment.

In the first experimental chapter (chapter 3)³ we demonstrate, in two separate mouse models, that leukocyte-vessel wall interactions are suppressed in the angiogenic environment of a tumor. We hypothesized that this escape of the tumor from immune surveillance is due to the production of growth factors by tumor cells. Tumors secrete high levels of angiogenic factors such as vascular endothelial cell growth factors (VEGFs) and fibroblast growth factors (FGFs). Previously, we have shown that these factors are responsible for downregulating endothelial adhesion molecules such as intercellular adhesion molecule-1/-2 (ICAM-1/-2), CD34^{13,14}, vascular cell adhesion molecule-1 (VCAM-1) and E-selectin^{15,16}. In addition, it has been established both *in vitro*¹⁷ and *in vivo*¹⁸ that suppressed adhesion molecule expression by angiogenic factors results in diminished leukocyte-vessel wall interactions. In the present study we demonstrate that when mice bearing a small ear tumor are treated with a humanized anti-VEGF antibody, the downregulation of leukocyte-vessel wall interactions inside the tumor is prevented (chapter 3)³, which is in support of our hypothesis. Interestingly, the present study also shows that a large tumor has a systemic effect: leukocyte-vessel wall interactions appear to be downregulated in ear microvessels of mice bearing a large tumor on

their flank (chapter 3)³. Apparently, a large tumor can produce such large quantities of angiogenic factors that their plasma concentration gets high enough to decrease endothelial adhesion molecule expression systemically. This hypothesis is supported by the observation that the systemic leukocyte count in TNF α treated mice bearing a large flank tumor is higher than in TNF α treated control mice (chapter 3)³, also suggesting a systemic effect of the tumor. Altogether, we have demonstrated that tumors have the capacity to prevent the formation of a proper anti-tumor immune response by the downregulation of endothelial adhesion molecules that are necessary for leukocyte-vessel wall interactions. These results are consistent with those described by other research groups^{6,19,20}. This downregulation of leukocyte-vessel wall interactions, that potentially leads to escape from immune surveillance and progression of the tumor, was found to be mediated by the production of angiogenic growth factors^{13,15}. A recent study in a large population of ovarian carcinoma patients elegantly demonstrated that the expression of VEGF in tumor tissue is negatively correlated with the presence of infiltrating T lymphocytes. Interestingly, the latter was found to be the strongest prognostic factor in ovarian carcinoma²¹.

Once a tumor is vascularized it can be infiltrated by leukocytes, which are indeed found in several solid cancers. The presence of these leukocytes is often the consequence of a specific immune reaction to the tumor itself, since the immune system is known to recognize tumor-associated antigens²². In cancer patients, specific cytotoxic T-lymphocytes recognizing tumor antigens have been reported and the presence of these cells is related to a better prognosis²³. In addition, antibodies to tumor-associated antigens produced by B-cells may also play a role in limiting tumor growth^{24,25}. We hypothesized that when angiogenesis downregulates the infiltration of leukocytes, inhibition of angiogenesis would potentially overcome endothelial cell anergy and lead to increased leukocyte infiltration in tumors. From *in vitro* experiments performed in our laboratory we know that the angiogenesis inhibitors platelet factor-4 (PF4) and thrombospondin-1 prevent the downregulation of adhesion molecules by angiogenic stimulation of cultured human umbilical vein derived endothelial cells²⁶. Similarly, angiostatin was found to enhance the expression of E-selectin²⁷, while the synthetic angiogenesis inhibitor SU5416 reversed the bFGF-mediated downregulation of ICAM-1, VCAM-1 and E-selectin²⁰. In chapter 4²⁸, we demonstrate that this phenomenon is shared by more angiogenesis inhibitors and that it has impact on *in vivo* leukocyte-vessel wall interactions and the subsequent formation of an immune infiltrate in a tumor. We found that the angiogenic inhibitors anginex, endostatin and angiostatin, and also the chemotherapeutic agent paclitaxel significantly stimulate leukocyte-vessel wall interactions by circumvention of EC anergy in tumor vessels *in vivo*. This was confirmed by *in vitro* studies on the expression of ICAM-1 and VCAM-1 on cultured endothelial cells at the protein and mRNA level. However, not all angiogenesis inhibitors tested showed the same inflammatory response. For treatment of cancer, the preferred agent should combine the strongest angiostatic activity with the most potent inflammatory effect. Apparently, these properties are not interdependent; e.g. TNP-470 and anginex differ largely in modulating immune property, while they equally inhibit angiogenesis and tumor growth²⁸, suggesting that the mechanisms of these effects are different. Anginex increased the expression of endothelial adhesion molecules on tumor endothelial cells both at mRNA and protein level, while TNP-470 had no such effect. Of the angiogenesis inhibitors used, anginex had the strongest inflammatory effect. It significantly increased leukocyte-vessel wall interactions in tumor vessels to levels found in healthy control animals. This, in turn, led to increased infiltration of leukocytes into the tumor in spite of the simultaneously occurring decrease in microvessel density. Interestingly, the inflammatory activity of the angiostatic compounds is shared with certain chemotherapeutic agents. It has been observed previously that chemotherapeutic compounds such as cyclophosphamide and paclitaxel can induce

leukocyte infiltration in tumors²⁹. The presently shown effects of paclitaxel on adhesion molecule regulation and leukocyte-vessel wall interactions (chapter 4)²⁸ supports the theory that certain chemotherapeutics are angiostatic and may be used at ultra low dosages for prolonged periods of time for the treatment of cancer. Except for cancer therapy, angiogenesis inhibition is also suggested to be a promising approach for the treatment of diseases with a chronic inflammatory component, such as rheumatoid arthritis and psoriasis²⁸, in which pro-inflammatory activity is undesirable. It is tempting to speculate that the good results with TNP-470 in arthritis models are due to its lack of pro-inflammatory activity.

The mechanisms by which angiogenesis inhibitors influence tumor angiogenesis on the one hand and the endothelial expression of adhesion molecules on the other remain unclear. Many research institutions perform mechanistic studies on angiogenesis inhibitors, but little is known about the endothelial cell components with which the inhibitors interact. The mechanisms underlying the suppression of angiogenesis appear to be complex and require several players³⁰. Several angiostatic compounds induce anoikis – or detachment of endothelial cells from the extracellular matrix – through blocking of adhesion^{31,32} or interference in cell cycle^{33,34}. In many of these examples exposure to the compound also forces EC to undergo apoptosis³⁵. Similarly, it is largely unknown how angiogenesis inhibitors can induce adhesion molecules. Although the molecular mechanisms are unknown as yet, it is suggested that growth signals to endothelial cells de-differentiate cells into a phenotype that is suited for proliferation and migration. In such a state leukocyte adhesion would not be a priority of endothelium.

The results described in chapter 4²⁸ of the present thesis are not in agreement with the recent observation by Chavakis and colleagues that angiostatin is a potent anti-inflammatory and anti-adhesive substance by interacting directly with leukocyte $\beta 1$ - and $\beta 2$ -integrins³⁶. Angiostatin was shown to inhibit leukocyte adhesion and transmigration *in vitro* and peritonitis-induced neutrophil emigration *in vivo*. This study was performed in the absence of a tumor, and in the *in vivo* experiments only one bolus injection of angiostatin was given 4.5 hours before measurement³⁶. In our mouse model, in which angiostatin was continuously administered via minipumps for 7 days, angiostatin did not influence leukocyte-vessel wall interactions outside the tumor or in the absence of a tumor. In addition, we found no effects of angiostatin on leukocyte adhesion molecule expression. Interestingly, Chavakis and colleagues noted that angiostatin containing kringle domains 1-4 (K1-4) or kringle 4 domain (K4) alone, but not kringles 1-3 (K1-3), binds to Mac-1 (a $\beta 2$ integrin binding to ICAM-1) on leukocytes. Neither type of angiostatin was shown to bind to another known ICAM-1 receptor, LFA-1³⁶. These results indicate that K1-3, the type of angiostatin used in our experiments, has a lower anti-adhesive effect on leukocytes than K1-4. Another recent paper by Yu and colleagues³⁷ describes that E-selectin, one of the adhesion molecules that are upregulated by anginex, is required for the angiostatic activity of endostatin. In a corneal micropocket assay they showed that endostatin does not inhibit angiogenesis in E-selectin deficient mice, while control mice showed 40% reduction in bFGF-induced vessel area upon treatment with endostatin. Interestingly, endostatin was not found to block E-selectin mediated leukocyte adhesion, suggesting that it does not interfere with E-selectin binding to its leukocyte ligands.

Although leukocyte infiltration in tumors is often associated with better prognosis and overall survival, it has now been shown by us and others^{3,7} that tumors have developed mechanisms to escape immune surveillance and frequently show only limited leukocyte infiltration³⁸. As a consequence, tumor immune responses are often not able to effectively destroy the tumor²². Leukocytes that do reach the tumor often remain localized in the tumor periphery or stroma and are often not able to exert strong antitumor effects³⁹. Moreover, it is known that infiltrated leukocytes

show impaired maturation due to exposure to the tumor microenvironment^{22,40}. By contrast, some leukocyte subsets have been described to promote proliferation and metastasis and, hence, tumor development. Tumor associated macrophages (TAM), for example, can exert protumor effects through the secretion of immunosuppressive cytokines, the release of free radicals like nitric oxide (NO) and hydrogen peroxide (rendering immune cells hyporesponsive or even apoptotic) and, last but not least, the secretion of angiogenic cytokines and enzymes (chapter 6)⁴¹. Therefore, when performing general immunotherapy, one has to keep in mind which leukocyte subsets are being recruited to the tumor and which subsets are desired at this location.

Beside tumor cells, endothelial cells and leukocytes, other cells may be involved in tumor development as well. A few years ago Pinedo and colleagues proposed the hypothesis that blood platelets are involved in tumor angiogenesis⁴². Quantitative and qualitative abnormalities of platelets are often found in cancer patients, due to increased or reduced thrombocytosis, spontaneous platelet aggregation or impaired adhesion⁴³. *in vitro* studies revealed that platelets are able to stimulate endothelial cell proliferation and tube formation on Matrigel⁴⁴. Many types of tumor cells are capable of platelet activation in a process termed tumor cell induced platelet aggregation⁴⁵ and activated platelets were shown to be present within the tumor vasculature of human sarcoma patients⁴⁶. Upon activation, platelets are known to release angiogenic factors like VEGF, bFGF and PDGF (platelet derived growth factor; for a recent review on this topic the reader is referred to the paper by Sierko and colleagues)⁴⁷. In several types of cancer, the systemic platelet count was found to significantly correlate with serum VEGF concentrations, whereas it did not correlate with plasma VEGF concentrations, indicating that platelets are an important source of VEGF⁴⁷. bFGF, like VEGF a constituent of platelet α -granules, is an important angiogenic factor inducing EC migration, proliferation and maturation. PDGF stimulates VEGF expression by endothelial cells and it is involved in vessel maturation by recruitment of pericytes⁴⁷. In addition, platelets have been shown to modulate degradation and further remodeling of basement membrane and extracellular matrix components, an essential step in the process of vessel formation⁴⁷. On the contrary, platelets also contain platelet factor-4⁴⁷ and thrombospondin-1⁴⁸, which are both endogenous inhibitors of angiogenesis. These findings illustrate that multiple cell types are involved in the process of new vessel formation, all of which have to be taken into account when studying tumor angiogenesis.

In AIDS-associated Kaposi's Sarcoma lesions, an angio-proliferative tumor, the level of leukocyte-vessel wall interactions appears to be high with prominent infiltration of monocytes and T-cells⁴⁹. This is in contrast to the situation in other tumors, where immune surveillance is low (see above). In one of our studies (chapter 5)⁵⁰, we show that the HIV-tat protein (a regulatory protein of HIV-1 released by infected cells) and the cytokine TNF α synergistically increase leukocyte-vessel wall interactions *in vitro* and *in vivo*. This, in turn, induces increased leukocyte adhesion (chapter 5)⁵⁰. The HIV-tat protein affects the interaction between monocytes and endothelial cells by upregulating the expression of β 2-integrin adhesion molecules and MMP9 in monocytes⁵¹. As a consequence, it is observed *in vitro* that adhesion of monocytes is increased, monocyte dependent damage of endothelial cell monolayers is enhanced, and invasive behavior of monocytes is promoted⁵²⁻⁵⁴. Our finding that HIV-tat and TNF α synergistically increase leukocyte adhesion to the endothelium *in vivo* may be explained by increased β 2-integrin expression on monocytes and T-cells by HIV-tat, and upregulation of ICAM-1, VCAM-1 and E-selectin on endothelial cells by both HIV-tat and TNF α (chapter 5)⁵⁰. It remains unclear why HIV-tat preferentially influences monocytes and T-cells, since β 2-integrins are expressed uniformly by leukocytes. Indeed, immunohistochemical studies of AIDS-associated Kaposi's Sarcoma lesions indicate infiltration of monocyte-macrophages together with CD4+ and CD8+ T-cells, whereas B-cells are rare or absent⁵⁵⁻⁶⁰. The co-operative activity of HIV-

tat and TNF α may explain the 20,000-fold increased incidence of Kaposi's Sarcoma in AIDS⁶¹; it may also contribute to other vascular defects in AIDS that are associated with increased leukocyte adhesion and extravasation into tissues, including cardiovascular diseases and atherosclerosis.

FUTURE DIRECTIONS

Angiogenesis inhibition is now considered to be a promising therapeutic approach for cancer. Approximately 40 angiostatic compounds are currently being tested in trials for human cancer therapy and the search for novel inhibitors is still ongoing^{62,63}. In chapter 4 we have tested some angiostatic compounds in our newly developed tumor model. It will be interesting to test some of the more recently developed angiostatic substances, such as Avastin and the SU- and ZD-compounds (like SU5416 (Semaxinib), SU6668 and SU11248 (all developed by Sugen), ZD4190 and ZD6474 (both developed AstraZeneca) and PTK787 (developed by Novartis)), for their action on leukocyte-vessel wall interactions in tumors.

As discussed in chapter 6, the role of a tumor immune infiltrate in cancer development has been debated frequently. Although often associated with improved prognosis and enhanced survival of cancer patients, several types of inflammatory cells have also been described to assist the tumor's capabilities to proliferate and metastasize. Therefore, it will be of great interest to investigate which leukocyte subsets are stimulated to interact with the vessel wall by angiostatic factors. To this purpose, leukocyte subsets can be isolated and labeled with a fluorescent probe *ex vivo*, and injected back into the mouse. Another interesting model to study leukocyte subsets is developed by von Andrian and colleagues⁶⁴. In order to track individual T-cells, this group has generated transgenic mice which express green fluorescent protein (GFP) uniformly in CD4+ and CD8+ T-cells. Since these mice were created on a C57Bl/6 background, we could use our B16F10 tumor model to study leukocyte trafficking to define whether CD4+ and CD8+ T-cells are stimulated to interact with the tumor vessel wall by angiostatic factors. Last but not least, cytotoxic T-lymphocytes (CTLs) specifically directed to the B16F10 tumor have been created by Melief and colleagues⁶⁵. It would be interesting to study homing of these CTLs in our B16F10 tumor model, with or without the use of angiostatic compounds.

For leukocyte-vessel wall interactions to occur, the expression and upregulation of endothelial adhesion molecules is necessary. In order to investigate which adhesion molecules are mainly affected and upregulated by angiostatic therapy, it will be interesting to label adhesion molecules such as ICAM-1, VCAM-1 or E-selectin *in vivo* and study their expression patterns, both in and outside a tumor, using fluorescence intravital and/or multiphoton laser scanning microscopy. Another possibility to study the effects of angiostatic therapy on adhesion molecules and leukocyte-vessel wall interactions in tumors is by the use of selectin or ICAM-1 knockout mice.

CONCLUSION

Inhibition of angiogenesis is a promising approach for treatment of cancer and various other diseases like diabetes, rheumatoid arthritis, and cardiovascular diseases. Angiostatic factors not only inhibit tumor vessel formation, inducing tumor starvation, but they also modulate leukocyte-vessel wall interactions, making the tumor more vulnerable to the immune system. A combination of angiostatic treatment with conventional immunotherapy strategies represents a promising way to improve cancer therapy.

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CHAPTER 7

Summary

Samenvatting

SUMMARY

This thesis describes the interplay between tumor angiogenesis and the immune system, with special emphasis on leukocyte-vessel wall interactions inside the tumor. In Europe and America, cancer is one of the most important causes of death. For the development of novel treatment strategies for cancer, it is necessary to fundamentally understand the process of tumor initiation, progression and metastasis. Angiogenesis is an important part of the last two. Besides allowing the tumor to grow and providing a route for metastasis, the presence of blood vessels in a tumor also enables various immune cells to enter the tumor and perform their particular immune function. As a result, the tumor can be infiltrated by leukocytes, a phenomenon observed in most solid cancers. Although frequently associated with better prognosis, these immune responses are often weak and not able to completely destroy a tumor, suggesting that tumors have developed mechanisms to escape immune surveillance. The aim of the present study was to investigate the relationship between angiogenesis and immunity in tumors and to explore ways of modulating this relationship in order to improve the prognosis of patients enhancing the activity of novel anti-cancer therapies.

Chapter 1 provides background information on several topics described in this thesis. First, an overview is given of both the angiogenic cascade and the process of leukocyte-vessel wall interactions. Subsequently, present knowledge on the influence of tumor angiogenesis on leukocyte-vessel wall interactions is discussed. Although angiogenesis enhances the possibilities for leukocytes to interact with the vessel wall inside a tumor, it has been shown before that exposure of endothelial cells to angiogenic factors (produced by tumor cells, such as VEGF and bFGF) downregulates adhesion molecule expression and, hence, leukocyte-vessel wall interactions *in vitro* and also *in vivo*. Moreover, an angiogenic environment hampers the upregulation of adhesion molecule expression by proinflammatory cytokines, a phenomenon called endothelial cell anergy. These tumor-regulated mechanisms are thought to help the tumor to escape immune surveillance. Finally, this chapter provides an introduction to the contents of the thesis.

For the studies presented in **chapters 2 and 3**, we have developed a new mouse model in which angiogenesis and leukocyte-vessel wall interactions can be quantified non-invasively both in and outside a tumor, allowing longitudinal analysis during tumor development and/or in the course of anticancer treatment. A small tumor was grown in the ear, and intravital microscopy was used to quantify *in vivo* the effects of the tumor on leukocyte-vessel wall interactions and on fluid dynamic conditions in microvessels in and outside the tumor. In the study described in **chapter 2**, we demonstrated in both a syngeneic mouse model (C57Bl/6 mice bearing murine B16F10 melanomas) and an allogeneic mouse model (nude mice bearing small human LS174T colon carcinomas) that leukocyte-vessel wall interactions are suppressed in microvessels within a small tumor, but not in healthy vessels outside the tumor. The reduction in leukocyte-vessel wall interactions in tumor vessels appeared to be caused by a decreased expression of adhesion molecules on endothelial cells, and not by suppression of adhesion molecules on leukocytes. FACS-analysis showed that isolated tumor endothelial cells have suppressed levels of ICAM-1 as compared to endothelial cells from normal mouse tissues. The study described in this chapter was the first to show a systemic effect of a large tumor: leukocyte-vessel wall interactions appeared to be downregulated in ear microvessels of mice bearing a large tumor on their flank. This systemic effect was less pronounced as compared to the situation within a tumor; leukocyte adhesion was only partially down regulated, while inside a tumor the level of adhesion was reduced to undetectable levels. Summarizing, the results described in this chapter show that tumors have evolved mechanisms to escape from immune surveillance.

In **chapter 3** we hypothesized that inhibition of angiogenesis would overcome this tumor escape from immunity. This was investigated both *in vivo* and *ex vivo* in our mouse models, applying

intravital microscopy, immunohistochemistry and qRT-PCR, and *in vitro* on cultured endothelial cells by means of FACS-analysis and qRT-PCR. The angiogenesis inhibitors anginex, endostatin and angiostatin, and also the chemotherapeutic agent paclitaxel were found to significantly stimulate leukocyte-vessel wall interactions by circumvention of endothelial cell anergy *in vivo*, i.e. by upregulation of endothelial adhesion molecules in tumor vessels. This was confirmed by *in vitro* studies on cultured endothelial cells at the protein and mRNA levels. The new angiostatic designer peptide anginex was most potent at overcoming endothelial cell anergy. The enhanced leukocyte-vessel interactions led to an increase in the numbers of tumor infiltrating leukocytes. While anginex inhibited tumor growth and microvessel density (MVD) significantly, the amount of infiltrating leukocytes (CD45), as well as the number of infiltrated CD8+ cytotoxic T-lymphocytes, was enhanced markedly. The results described in this chapter indicate that not all angiogenesis inhibitors tested showed the same inflammatory response. The angiogenesis inhibitor TNP-470 was not able to increase leukocyte-vessel wall interactions in tumor vessels. Treatment of mice with the chemotherapeutic agent cyclophosphamide did induce an increase in leukocyte-vessel wall interactions in tumor vessels, but this effect did not reach the level of significance. The results presented in this chapter suggest that immunotherapy strategies can be improved by combination with anti-angiogenic and chemotherapeutic agents.

In AIDS-associated Kaposi's sarcoma lesions, an angio-proliferative tumor, the level of leukocyte-vessel wall interactions appears to be high with prominent infiltration of monocytes and T-cells. This is in contrast to the situation in other tumors, where immune surveillance is low (see above). In the study described in **chapter 4**, we show that the HIV-1 Tat protein (a regulatory protein of HIV-1 released by infected cells) and TNF α (a cytokine increased in sera and tissues of HIV-1-infected patients) synergistically stimulate the adhesion of leukocytes to endothelial cells *in vitro* as well as *in vivo*. This effect is selectively mediated by HIV-1 Tat, since HIV-1 Nef, another HIV-1 regulatory protein, and the HIV-1 envelope protein gp41, had no effect. *In vitro* adhesion assays with peripheral blood mononuclear cells (PBMC) and quantitative cell type analysis of adherent cells by FACS demonstrated that HIV-1 Tat selectively activates the adhesion of T-cells and monocytes, but not of B-cells. Intravital microscopic studies in mice confirmed the synergistic activity of HIV-1 Tat and TNF α on leukocyte adhesion to the endothelium *in vivo*. These data indicate that HIV-1 Tat in combination with TNF α may contribute to the vascular damage and cardiovascular diseases observed in AIDS patients, and also to the prominent extravasation of T-cells and monocytes which is a key process in the formation and progression of Kaposi's sarcoma lesions.

The role of a tumor immune infiltrate in cancer progression and metastasis has frequently been debated. Although often considered to be associated with improved prognosis and leading to enhanced survival of cancer patients, inflammatory cells have also been described to assist the capability of the tumor to progress, proliferate and metastasize. Tumor associated macrophages (TAMs), for example, have been shown to be symbiotically related to tumor cells. Tumor cells recruit TAMs and provide them with survival factors, while TAMs in turn produce a variety of angiogenic factors in response to the tumor microenvironment. **Chapter 5** reviews the research on the composition of immune infiltrates in tumors, and on the angiogenic and angiostatic properties of the infiltrated immune cells. Special emphasis is on the angiogenesis-associated activities of TAMs. Although little is known on the angiostatic properties of TAMs, they are reported to produce several angiostatic compounds, such as IL-12, IL-18 and matrix metalloproteinase-12 (MMP-12). In contrast, more research has focused on their angiogenic properties (a.o. production of IL-1, VEGF, IL-8, bFGF, TNF α and MMP-9), which will hopefully raise new opportunities to counteract these activities in order to treat cancer. The development of immune and gene therapies using TAMs to mediate tumor

cytotoxicity or to deliver gene constructs is also discussed. The ability of macrophages to infiltrate tumors can be used to develop immunotherapeutic approaches for cancer treatment. Since immunotherapy has so far not been as effective as anticipated, a combination therapy in which angiostatic agents are used as well is put forward as a novel strategy to treat cancer.

In conclusion, inhibition of angiogenesis is a promising approach for treatment of cancer and various other diseases like diabetes, rheumatoid arthritis, and cardiovascular diseases. Angiostatic factors not only inhibit tumor vessel formation, inducing tumor starvation, but they also modulate leukocyte-vessel wall interactions, making the tumor more vulnerable to immune attack. A combination of angiostatic treatment with conventional immunotherapy strategies represents a promising way to improve cancer therapy.

SAMENVATTING

In dit proefschrift wordt de wisselwerking tussen tumor angiogenese en het immuunsysteem beschreven. De nadruk ligt hierbij op de interacties die leukocyten (witte bloedcellen) aangaan met de bloedvat wand. In de westerse wereld is kanker één van de voornaamste doodsoorzaken. Daarom is het belangrijk om de verschillende processen die betrokken zijn bij het ontstaan van een tumor zo goed mogelijk te begrijpen, en deze kennis te gebruiken voor het ontwikkelen van nieuwe therapieën tegen deze ziekte. De vorming van nieuwe bloedvaten die een tumor van bloed gaan voorzien (angiogenese) is noodzakelijk voor de groei van de tumor en maakt tevens metastasering van de tumor mogelijk. Daartegenover staat dat de aanwezigheid van bloedvaten in een tumor er ook toe leidt dat cellen uit het immuunsysteem in de tumor kunnen doordringen. Veel tumoren zijn dan ook geïnfiltrerd met leukocyten. Echter, de immuunrespons van deze leukocyten tegen de tumor blijkt vaak erg zwak te zijn en niet in staat om de tumor te vernietigen. Dit suggereert dat tumoren mechanismen hebben ontwikkeld om te ontsnappen aan het immuunsysteem. Het doel van de studies die in dit proefschrift zijn beschreven was dan ook om de relatie te onderzoeken die bestaat tussen tumor angiogenese en de werking van het immuunsysteem in tumoren, om via deze weg bij te dragen aan de ontwikkeling van een nieuwe behandelmethode tegen kanker.

Het **eerste hoofdstuk** verschaft achtergrondinformatie omtrent de verschillende onderwerpen die in dit proefschrift aan bod komen. Allereerst wordt er een overzicht gegeven van de angiogenese cascade en het proces van leukocyt-vaatwand interacties. Hoewel tumor angiogenese in principe de mogelijkheid voor leukocyten om een interactie aan te gaan met de vaatwand binnen de tumor vergroot, is ook bekend dat de blootstelling van endotheelcellen aan angiogene factoren (geproduceerd door tumorcellen, zoals VEGF en bFGF) de expressie van adhesiemoleculen op het endotheel verlaagt en daarmee zowel *in vitro* als *in vivo* leidt tot een daling in leukocyt-vaatwand interacties. Bovendien blijkt de opregulatie van endotheliale adhesiemoleculen door pro-inflammatoire cytokinen geremd te worden in een angiogeen milieu (ook wel endotheelcel anergie genoemd). Bovenstaande mechanismen spelen waarschijnlijk een rol bij het ontsnappen van een tumor aan het immuunsysteem. Het eerste hoofdstuk wordt afgesloten met een overzicht van de inhoud van het proefschrift.

Voor de experimenten die beschreven staan in **hoofdstukken 2 en 3** hebben we een nieuw muismodel ontwikkeld, waarin angiogenese en leukocyt-vaatwand interacties op een niet-invasieve wijze bestudeerd kunnen worden, zowel binnen als buiten een tumor. Met behulp van intravitaal microscopie zijn leukocyt-vaatwand interacties *in vivo* in venulen in de huid van een muizenoor bestudeerd, zowel in nieuw-gevormde vaten in de tumor als in gezonde vaten buiten de tumor. In het tweede hoofdstuk worden experimenten beschreven die zijn uitgevoerd in een syngene muis model (C57Bl/6 muizen met een B16F10 melanoom) en in een allogene muismodel (Swiss/nude muizen met een humaan LS174T colon carcinoom). De bevindingen tonen aan dat het niveau van leukocyt-vaatwand interacties in tumorvaten significant lager is dan in gezonde vaten buiten de tumor. De verminderde leukocyt-vaatwand interacties in tumor vaten bleken veroorzaakt te worden door een verlaagde expressie van adhesiemoleculen op de endotheelcellen, en niet op de leukocyten zelf. Analyse met behulp van FACS liet namelijk zien dat de ICAM-1 expressie op tumor endotheelcellen lager is dan op endotheelcellen verkregen uit gezond muizenweefsel. Daarnaast werd in deze studie voor de eerste keer aangetoond dat een grote tumor een systemisch effect heeft; in muizen met een grote tumor op de flank was het aantal leukocyt-vaatwand interacties in bloedvaten in de oorhuid lager dan in gezonde muizen. Dit systemisch effect was wel minder uitgesproken dan de situatie in de tumor zelf; de adhesie van leukocyten aan de vaatwand was slechts gedeeltelijk verlaagd, terwijl in tumor vaten het niveau van leukocyten adhesie bijna tot het

nulpunt gedaald was. We concluderen uit deze resultaten dat tumoren mechanismen hebben ontwikkeld om te ontsnappen aan het immuunsysteem.

Indien de tumor angiogenese mede verantwoordelijk is voor het feit dat tumoren het immuunsysteem ontwijken, dan kan remming van die angiogenese er wellicht voor zorgen dat tumoren weer detecteerbaar worden voor het immuunsysteem. Dit is dan ook de hypothese waarmee we tijdens de experimenten die beschreven staan in **hoofdstuk 3** hebben gewerkt. Zowel *in vivo* als *ex vivo* (intravitaal microscopie, immunohistochemie en qRT-PCR) en *in vitro* (FACS analyses en qRT-PCR) is gekeken naar de effecten van angiogenese remming op leukocyt-vaatwand interacties en de expressie van adhesiemoleculen in tumor bloedvaten. De angiogeneseremmers anginex, endostatine en angiostatine, maar ook het chemotherapeuticum paclitaxel stimuleerden op significante wijze de leukocyt-vaatwand interacties in tumor vaten door middel van opregulatie van de adhesiemolecuul expressie op endotheelcellen. Dit werd bevestigd door *in vitro* experimenten met gekweekte endotheelcellen, zowel op eiwit als mRNA niveau. De nieuwe angiogeneseremmer anginex was het beste in staat de endotheelcel anergie op te heffen, wat resulteerde in een toegenomen infiltratie van leukocyten (CD45) en CD8+ cytotoxische T-cellen in behandelde tumoren. De resultaten laten ook zien, dat niet iedere angiogeneseremmer in staat is leukocyt-vaatwand interacties in tumoren te doen toenemen (zoals geldt voor TNP-470). Ook het chemotherapeuticum cyclophosphamide slaagde er niet in de leukocyt-vaatwand interacties in tumoren significant te verhogen. De resultaten beschreven in dit hoofdstuk laten echter wel zien dat immuuntherapie tegen kanker wellicht verbeterd kan worden door te combineren met remming van angiogenese.

In Kaposi's Sarcoma, een vorm van kanker die vaak wordt gezien in AIDS-patiënten, is het niveau van leukocyt-vaatwand interacties hoog en wordt veelal een grote infiltratie gezien van monocyten en T-cellen, dit in tegenstelling tot de situatie in veel andere tumoren. In de experimenten beschreven in het **vierde hoofdstuk** laten we zien dat het HIV-1 Tat proteïne (een regulator eiwit van HIV-1, afgegeven door geïnfecteerde cellen) en TNF α (een inflammatoir cytokine dat verhoogd is in het serum en in de weefsels van HIV-1 geïnfecteerde individuen) op synergistische wijze de adhesie van leukocyten aan het endotheel stimuleren, zowel *in vitro* als *in vivo*. Dit effect kan toegeschreven worden aan HIV-1 Tat, aangezien twee andere eiwitten, HIV-1 Nef en HIV-1 gp41, geen effect op leukocyt-vaatwand interacties bleken te hebben. Met behulp van *in vitro* adhesie experimenten met mononucleaire perifere bloedcellen gevolgd door een FACS analyse, is aangetoond dat HIV-1 Tat bovendien selectief de adhesie van T-cellen en monocyten stimuleert, maar niet van B-cellen. Intravitaal microscopie bevestigde de synergistische activiteit van HIV-1 Tat en TNF α op leukocyt-vaatwand interacties *in vivo*. Deze data laten zien dat HIV-1 Tat in combinatie met TNF α een bijdrage zou kunnen leveren aan de cardiovasculaire ziekten die vaak optreden in AIDS-patiënten, en daarnaast ook aan de extravasatie van T-cellen en monocyten die een belangrijke rol speelt bij het ontstaan en de progressie van lesies behorend bij Kaposi's Sarcoma.

De rol van een immuuninfiltraat tijdens de progressie en metastasering van kanker wordt tot op de dag van vandaag betwist. Vaak wordt de aanwezigheid van zo'n infiltraat geassocieerd met een verbeterde prognose en een verhoogde overlevingskans voor kanker patiënten. Echter immuuncellen worden ook wel beschreven als cellen die de tumor assisteren met betrekking tot proliferatie en metastasering. Tumor geassocieerde macrofagen (TAMs) zijn bijvoorbeeld op symbiotische wijze verbonden met tumorcellen; tumorcellen rekruteren TAMs en zorgen voor hun overleving, terwijl TAMs op hun beurt diverse angiogene factoren produceren die de tumor helpen zich verder te ontwikkelen. **Hoofdstuk 5** is een review van de huidige stand van zaken in het onderzoek naar de samenstelling van een immuuninfiltraat in tumoren en naar de angiogene en

anti-angiogene eigenschappen van geïnfilteerde immuuncellen. Speciale aandacht wordt besteed aan de met angiogenese geassocieerde activiteiten van TAMs. Er is weinig bekend over de anti-angiogene eigenschappen van TAMs, maar we weten wel dat ze verschillende anti-angiogene factoren produceren, zoals IL-12, IL-18 en matrix metalloproteinase-12 (MMP-12). Er is meer bekend over de angiogene eigenschappen van TAMs (zoals de productie van IL-1, VEGF, IL-8, bFGF, TNF α en MMP-9). Dergelijke kennis zou bij kunnen dragen aan de ontwikkeling van nieuwe mogelijkheden om deze eigenschappen tegen te gaan en zo te komen tot een nieuwe methode om kanker te behandelen. De ontwikkeling van immuun- en gentherapieën waarbij gebruik wordt gemaakt van TAMs wordt ook in dit hoofdstuk beschreven. Het feit dat macrofagen in staat zijn tumoren te infiltreren zou gebruikt kunnen worden om nieuwe immuuntherapeutische benaderingen te ontwikkelen voor de behandeling van kanker. Echter, immuuntherapie is tot op heden niet zo effectief gebleken als tevoren gedacht; wellicht zou een gelijktijdige behandeling met angiogese remmers hier een oplossing kunnen bieden.

De remming van angiogenese is een veelbelovende manier om kanker te behandelen, maar zou ook toepasbaar kunnen zijn voor andere ziekten zoals diabetes, reumatoïde artritis en hart- en vaatziekten. In een tumor remmen anti-angiogene factoren niet alleen de vorming van nieuwe bloedvaten (om zodoende de tumor uit te hongeren), maar ze maken de tumor ook meer toegankelijk voor het immuunsysteem, aangezien ze een stimulerend effect hebben op leukocyt-vaatwand interacties. De combinatie van angiogeneseremmers en conventionele immuuntherapie is een veelbelovende manier om de effectiviteit van de behandeling van kanker te verhogen.

Curriculum Vitae

CURRICULUM VITAE

Anita Elizabeth Maria Dirkx was born on February 8th 1976 in Eindhoven, the Netherlands. She completed her secondary education at the van Maerlantlyceum in Eindhoven in 1994. In the same year, she started her academic education at Maastricht University, where she studied Health Sciences with Biological Sciences as specialization. She conducted her first internship at the department of Immunology at Maastricht University under supervision of Dr. A. Duijvestijn. During this 9 month period she studied the role of brain endothelial cells in the induction of T-cell proliferation. In September 1999 she left Maastricht for her second internship at Organon N.V. in Oss, where she was supervised by Dr. M. Boots and Dr. J. Dulos. She investigated the effects of DHEA (dehydroepiandrosteron) on the progression of rheumatoid arthritis. In November 2000 she obtained her MSc degree and from February 2000 until February 2005 she was appointed as a PhD student at the faculty of Medicine at the University Hospital Maastricht. Under supervision of Prof. Dr. A.W. Griffioen, Dr. M.G.A. oude Egbrink and Prof. Dr. J. Wagstaff she worked at the departments of Internal Medicine, Pathology and Physiology to investigate leukocyte-vessel wall interactions in tumors. On February 1st 2005 she has joined the clinical documentation group at Organon N.V., to continue her career as Medical Writer.

Dankwoord

*...ik moet er niet aan denken dat ik
dàt allemaal alleen had moeten doen...
(Herman Finkers, 'Ik Jan Klaasen')*

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maar...' was een opmerking die vaak uit jouw mond kwam wanneer ik weer eens in een opvoedkundig dilemma beland was. Viviane, jij hebt me de beginselen van het werken met muizen bijgebracht. En als de microscoop even nèt niet deed wat ik wilde, bracht het 'Viviane-knopje' vaak uitkomst... Prof. Dr. D.W. Slaaf, beste Dick, bedankt voor je kritische blik op mijn proefschrift; bedankt ook dat je zitting wilde nemen in mijn beoordelingscommissie. Lieve Marc (Eline, Rosa en Myrthe), bedankt voor je vriendschap en de vrolijke noot die je aan mijn tijd in Maastricht hebt gegeven. Ik hoop dat we nog erg vaak met zijn achten op vakantie gaan! Sietze, Remco en Kim, nog veel succes met jullie eigen promotie, natuurlijk onder Marc zijn bezielende leiding. Wim, als computer-leek kon ik altijd bij je terecht; bedankt! Marijke en Imke, jullie sprankelende aanwezigheid was als een frisse wind door het lab, bedankt hiervoor en succes met jullie eigen promotie! Andries, Pascal, Ward, Birgit en Richard bedankt voor alle gezellige momenten.

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List of Publications

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Full papers

- 1 Dirkx AEM, oude Egbrink MGA, Wagstaff J, Griffioen AW. Monocyte/macrophage infiltration in tumors: modulators of angiogenesis. *J. Leukocyte Biol.* 2006 (accepted).
- 2 Dirkx AEM, oude Egbrink MGA, Castermans K, van der Schaft DWJ, Thijssen VLJL, Dings RPM, Kwee L, Mayo KH, Wagstaff J, Bouma-ter Steege JCA, Griffioen AW. Anti-angiogenesis therapy can overcome endothelial cell anergy and promote leukocyte-endothelium interactions and infiltration in tumors. *FASEB J.* 2006 (accepted).
- 3 Matzen K, Dirkx AEM, oude Egbrink MGA, Speth C, Götte M, Ascherl G, Griffioen AW, Stürzl M. HIV-1 Tat increases the adhesion of monocytes and T-cells to the endothelium in vitro and in vivo: implications for AIDS-associated vasculopathy. *Virus Research*, 2004;104 (2): 145-155.
- 4 Dirkx AEM, oude Egbrink MGA, Kuijpers MJE, van der Niet ST, Heijnen VTh, Bouma-ter Steege JCA, Wagstaff J, Griffioen AW. Tumor angiogenesis modulates leukocyte-vessel wall interactions in vivo by reducing endothelial adhesion molecule expression. *Cancer Research*, 2003;63 (9), 2322-2329.

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- 5 Dirkx AEM, oude Egbrink MGA, Castermans K, Thijssen VLJL, van der Schaft DWJ, Kwee L, Bouma-ter Steege JCA, Griffioen AW. Anti-angiogenesis therapy can overcome endothelial cell anergy and promote leukocyte-vessel wall interactions and infiltration in tumors. First European Conference on Tumor Angiogenesis and Antiangiogenic Theory. *Angiogenesis* 2004;7 (S1), 1-53.
- 6 Dirkx AEM, oude Egbrink MGA, Castermans K, Thijssen VLJL, van der Schaft DWJ, Kwee L, Bouma-ter Steege JCA, Griffioen AW. Anti-angiogenesis therapy can overcome endothelial cell anergy and promote leukocyte-endothelium interactions in tumors. *The Netherlands Society for Microcirculation and Vascular Biology (MIVAB) 2004 (oral presentation).*
- 7 Dirkx AEM, oude Egbrink MGA, Castermans K, Thijssen VLJL, van der Schaft DWJ, Kwee L, Bouma JCA and Griffioen AW. Anti-angiogenesis therapy can overcome endothelial cell anergy and promote leukocyte-vessel wall interactions and infiltration in tumors. *J Vasc Res* 41(S2): 22, 2004.
- 8 Dirkx AEM, oude Egbrink MGA, Kuijpers MJE, van der Niet ST, Heijnen VTh, Bouma-ter Steege JCA, Wagstaff J, Griffioen AW. Tumor angiogenesis modulates leukocyte-vessel wall interactions in relation to tumor size. *9th Euregional Oncology Meeting 2002 (oral presentation).*
- 9 Dirkx AEM, oude Egbrink MGA, Kuijpers MJE, van der Niet ST, Heijnen VTh, Bouma-ter Steege JCA, Wagstaff J, Griffioen AW. Tumor angiogenesis modulates leukocyte-vessel wall interactions in relation to tumor size. *The Dutch Society for Microcirculation and Vascular Biology (MIVAB) 2002 (oral presentation).*

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- 11 Dirkx AEM, oude Egbrink MGA, Kuijpers MJE, Tromp SC, van der Niet ST, Wagstaff J, Griffioen AW. Tumour angiogenesis modulates leukocyte-vessel wall interactions. *Benelux & Dutch Society for Microcirculation 2001 (oral presentation)*.
- 12 AEM Dirkx, MGA oude Egbrink, MJE Kuijpers, ST van der Niet, J Wagstaff and AW Griffioen. Reduced leukocyte adhesion in vivo in the presence of a tumor. *Proceedings of the 7th World Congress for Microcirculation, Sydney, 2001*.