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Review

Tumor Vascular Endothelium: Barrier or Target in Tumor Directed Drug Delivery and Immunotherapy

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The therapy of solid tumors with conventional chemotherapeutics, drug delivery preparations and immunomodulatory agents directed against the tumor cells is corrupted by a major barrier presented by the tumor vasculature. Permeability of the tumor blood vessels for transport of small molecules and macromolecular drug-carrier conjugates is only sufficient in the blood vessels at the tumor-host interface. Downregulation of the expression of adhesion molecules, required for the facilitation of immune cell recruitment, by the tumor vascular endothelium results in an escape of the tumor from host defence.

New therapeutic approaches for the treatment of solid tumors are aimed at the tumor vasculature, either at the endothelial cells themselves or at basement membrane or tumor stroma components. Angiogenesis can be directly blocked with angiogenesis inhibitors, while angiogenesis related factors can serve as tumor vasculature specific epitopes for drug delivery strategies. Some glycoproteins expressed by tumor endothelial cells or present in the basement membrane and tumor stroma are also potential tumor selective targets. Therapeutic modalities that are suitable for site specific delivery are agents that increase tumor accumulation of (targeted) chemo/radiotherapeutics through increasing tumor vascular permeability. The observation that for tumor growth the blood supply is a limiting factor, led to the development of strategies to inhibit angiogenesis or block the tumor blood flow. Manipulation of the expression of endothelial cell adhesion molecules by selectively delivering modulatory agents at or in the tumor vascular endothelial cells may induce (bispecific antibody mediated) host defense activity directed against the tumor cells.

KEY WORDS: tumor vascular endothelium; targeting; drug delivery; immunotherapy; angiogenesis.

INTRODUCTION

In the therapy of non-operable solid tumors, the approach so far has been to directly attack tumor cells. The current cytostatic agents mainly interfere with processes involved in cell growth while the aim in immunotherapy with e.g. cytokines is to make immune effector cells selectively attack the tumor cells. To lower toxicity and increase therapeutic efficacy, drug delivery systems for anti-tumor therapy have been developed in the last decennia (1–3). These drug delivery systems, including soluble polymeric carriers and particle type carriers such as (immuno)liposomes and microspheres, are often also directed against epitopes present on tumor cells and carry drugs interfering with tumor cell growth. In all cases, the pharmacologically active agents or effector cells have to cross the tumor blood vessel wall consisting of endothelial cells and basement membrane. Especially in drug delivery strategies in which macromolecular (particulate) carriers or cells are used to increase treatment selectivity and effectiveness, the endothelial barrier forms a major obstacle.

A new area of research is aimed at procedures to decrease the particular barrier function or focusses on avoiding the necessity for carrier systems of crossing the tumor vascular wall. The vascular endothelium, basement membrane and tumor stroma may contain potential tumor specific targets. Strategies under investigation directed to these potential targets are aimed at interfering with blood vessel permeability, angiogenesis or tumor blood supply, or at manipulating endothelial cell mediated facilitation of immune effector cell movement into the tumor tissue.

Normal Blood Vessel Organization

Vascular endothelium is the sheet of thin squamous epithelial cells lining the blood vessels. The great majority of blood vessels consist of continuous endothelium, whereas endothelium in visceral capillaries are fenestrated. In organs such as

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ABBREVIATIONS: CTL: cytotoxic T lymphocyte; ECM: extracellular matrix; a/bFGF: acidic/basic fibroblast growth factor; ICAM: intercellular adhesion molecule; LFA: lymphocyte function-associated antigen; MoAb: monoclonal antibody; ScFv: single chain Fv; TGFβ: transforming growth factor β; TNFα: tumor necrosis factor α; VCAM: vascular cell adhesion molecule; VEGF: vascular endothelial growth factor.

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hematopoietic tissues and liver, the endothelium is discontinuous.

The cardiovascular tissues are arranged in concentric layers (tunics) that contain, from the lumen outwards, the endothelium, basal lamina (basement membrane), subendothelial connective tissue and internal elastic lamina (tunica intima), muscular cells, elastic lamellae and external elastic lamina (tunica media), and connective tissue (tunica adventitia). The fine microfibrillar basal lamina which is produced by the endothelium itself, is mainly made up of collagen type IV, V and VIII, laminin, heparan sulphate, chondroitin sulphate proteoglycans, entactin and sialoglycoproteins. The connective tissue consists of collagens, proteoglycans, fibronectin, and cells. All blood vessels with diameters smaller than 100 μm (arterioles, capillaries and their emerging venules as well as arteriovenous anastomoses) are referred to as the microvasculature. Unlike large vessels that occur as rather isolated anatomical entities, the microvasculature appears as an integral part of the tissue they supply. Under the influence of various physiological conditions, the basic layers of the blood vessel walls have undergone characteristic segmental differentiations. Mechanical factors act primarily on the large vessels, metabolic factors mostly affect capillaries and pericytic venules which are involved in the blood-tissue exchanges (4).

Vascular endothelium is clearly multi-functional. It plays a central role in the distribution of low and high molecular weight substances to tissue by being selectively permeable to these molecules. Furthermore, it is involved in hemostasis (vascular tone) and thrombosis through its ability to monitor the local environment and produce biologically active factors in response. Through complex interactions with inflammatory cells, chemoattractants and extracellular matrix, the endothelial cells are capable of recruiting the required immune cells into inflammatory sites. Endothelial cells are actively participating in vascular remodelling through the production of growth-promoting and growth-inhibiting substances (4). Besides a clear-cut role for endothelium in the above described processes, even more complex functions may occur. For instance, endothelial cells are capable of actively lysing tumor cells when exposed to low levels of cytokines (5), albeit only observed in vitro so far. Furthermore, an accessory function in the stimulation of T cells mediated by LFA-1/ICAM-1 and CD2/LFA-3 interactions has been proposed for endothelial cells (6).

Endothelium as a Permeability Barrier

The basal lamina on which the endothelium rests, and the endothelium itself constitute the main permeability barrier. Transport of solutes across the endothelium depends on the interactions of the transported molecules with the endothelial cell membrane. These interactions are not only dictated by intrinsic properties of the endothelium or the inherent leakiness of endothelial intercellular junctions, but also by the endothelial cell membrane surface electrostatic charge and composition of the subendothelial matrix proteins (7). The transition zone between plasma and endothelium is composed of adsorbed plasma proteins and glycosaminoglycans, oligosaccharide moieties of cell membrane glycoproteins and glycolipids, as well as sialoconjugates (4). This endocapillary molecular coat is presumed to be involved in phenomena such as cell adhesion, stabilisation of receptors, cellular protection and regulation of extravasation rates (8).

Endothelial cells contain a large population of membraneous vesicles involved in transcytosis, the transport of substances across the endothelium (Figure 1). Instead of heading towards the lysosomes, the vesicles discharge their content by exocytosis on the opposite side of the cell. Vesicle density varies significantly from one type of capillary to another and sometimes the vesicles form transient transendothelial channels through which plasma and interstitial fluid can communicate directly (9).

In addition to vesicles and channels, the endothelium of most visceral capillaries contain transcellular circular openings called fenestrae. Fenestrae lack a lipid bilayer, but do contain a diaphragm consisting of heparan sulphate proteoglycans, which give the diaphragms a negative charge. In most endothelia coated pits are present. In fenestrated and continuous capillaries, the coated pits contain a characteristically high density of anionic sites. In endothelium of some vascular beds, it has been shown that the coated pits are involved in receptor mediated endocytosis of macromolecules (9). Receptor mediated endocytosis (Figure 1) is often identified with the binding of a ligand to a specific cell surface receptor, followed by internalization of the ligand-receptor complex. Proteins and other molecules entering the receptor mediated or fluid phase endocytotic route are destined for the endothelial cells themselves. Transcytosis occurs for instance in the case of lectins that bind to carbohydrate moieties on the cell surface and of cationized molecules that bind to negatively charged cell surface components (10,11). In addition, macromolecules like native, acetylated and oxidized LDL (12), albumin and cationized or glycosylated derivatives (11,13), anionic ferritin (9), hCG and antibodies against LH/hCG receptor (14) and iron-transferrin (15) are subject of transcytosis through endothelial cells.

The Role of Endothelium in Leukocyte Recruitment

Normal leukocyte recirculation and recruitment during inflammatory processes are mediated by a complex array of adhesion molecules and chemoattractants (16). The dynamic processes of leukocyte tethering and rolling, adhesion and extravasation (Figure 2) are based on a coordinated expression or change of affinity of selectins, mucins, integrins and members of the immunoglobulin super family (Ig SF) both on leukocytes and endothelium. Initial attachment and rolling on endothelium is in most cases mediated by selectins and their ligands, mucins. As the leukocytes role, they may become activated through which they can enter the next step, tight adhesion. This step is mediated by members of the β_1 and β_2 integrin families and of the Ig SF, as is the transendothelial migration process. The actual movement of the cells into the inflamed site requires enzymes capable of degrading the basement membrane, cell-extracellular matrix interactions for locomotion and the presence of chemoattractants (17).

The use of combinations of adhesion molecules depends on variables like immune cell subset and activation status of both immune cells and endothelial cells. For example, in in vitro experiments it has been shown that in the case of resting T lymphocytes and resting endothelium, especially ICAM-1/LFA-1 interactions are used for binding. Upon endothelial cell activation, VCAM-1 and VLA-4 play an important role in

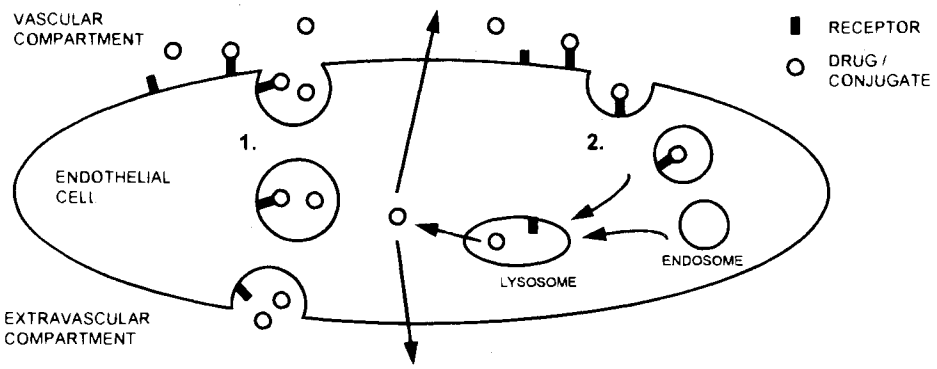

Fig. 1. Possible routes of handling of xenobiotics by vascular endothelial cells. 1. Receptor-mediated or fluid-phase transcytosis. Transported molecules will end up at the abluminal side of the endothelial lining. 2. Receptor-mediated endocytosis. Transported molecules will end up in the lysosomes, from which they can diffuse into the cytoplasm of the endothelial cell. They either stay in the cell or redistribute into the circulation or into the tissue beneath the endothelial lining.

the binding. Binding of activated T cells to either resting or activated endothelium could be partially blocked by antibodies against LFA-1 and only modestly by antibodies against ICAM-1. Regardless of the activation status of the T cells and the endothelium, VCAM-1 was never found to function during transendothelial migration. ICAM-1 on the other hand played a major role during transendothelial migration under all conditions examined (18).

Angiogenesis and Tumor Blood Vessel Organization

Already in the early 1970s it had become apparent that large numbers of cells within a solid tumor could only exist by virtue of the formation of new capillaries, a process called angiogenesis (19). It was observed that during the avascular phase of tumor growth the size of experimental tumors was limited. Also in the clinic, in occasionally detected carcinoma in situ, no capillaries were found. After an undefined period of time, new capillaries started to form and the tumor mass increased (19).

Angiogenesis involves an elaborate interplay between cells, soluble factors and extracellular matrix components. The role of the endothelial cells is multiple: they have to degrade basement membrane, migrate, proliferate and form new capillary tubes. Turnover time of normal endothelial cells is estimated to be in the range of 1000 days or more. In contrast, in tumors endothelial cells can grow with a turnover time of only 4–5 days (20). At least 20 angiogenic peptides have been found (21). They influence the endothelial cells either directly to perform any of the above mentioned activities (e.g. aFGF, bFGF and VEGF) or indirectly by inducing host cells to produce endothelial cell growth factors (e.g. TNFα and TGFβ) (22). The angiogenic peptide VEGF (also called VPF, vascular permeability factor) can also indirectly induce angiogenesis by causing leakage of plasma proteins including fibrinogen. Fibrinogen clots to form an extravascular fibrin gel matrix that stimulates neovascularization and subsequent deposition of mature connective tissue (23).

Overall, the vasculature within tumor tissue is highly disordered with numerous vascular shunts. The density of functional vessels is lower and vascular diameters are irregular and slightly higher compared to normal tissue (24). Wide interendothelial junctions can be observed, as well as large numbers of fenestrae and transendothelial channels, and discontinuous or absent basement membranes (25). The heterogeneity in angiogenic peptide expression by the tumor cells and other cellular components within the tumor (26), is most likely the reason for regional differences in endothelial cell kinetics and functional heterogeneity of the tumor vasculature.

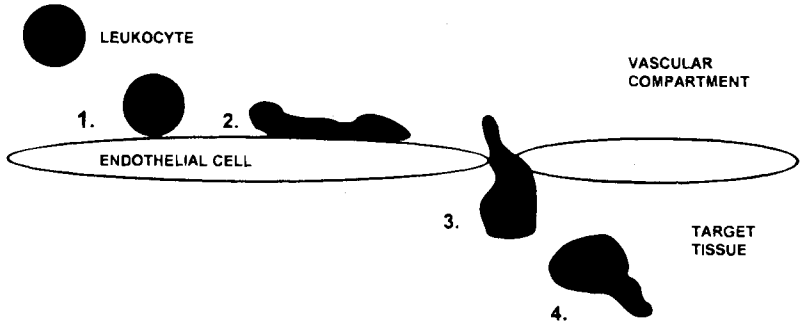

Fig. 2. Processes involved in the tissue homing of leukocytes. After initial attachment and rolling (1), the leukocyte will be activated followed by arrest and firm adherence (2). The next step is transendothelial migration (3), after which the cell has to find its way into the tissue by migration through the extracellular matrix (4).

Angiogenesis related factors influence both vascular permeability and immune cell recruitment. Permeability of tumor blood vessels is higher than in normal tissue. There is, however, a large variation within a tumor and between various tumor types. Furthermore, *in vivo* leukocyte rolling on and adhesion to angiogenic vessels in tumor tissue was completely absent. In tumor vessels that were not under the influence of angiogenic stimuli, only rolling of leukocytes was significantly reduced (27).

Tumor Vascular Endothelium as a Barrier

In tumor therapy drugs are most likely to be administered intravenously. Therefore, they have to find their way via the systemic circulation into the tumor tissue. The drugs as well as the immune cells in (targeted) immunotherapy have to cross the endothelial lining of the tumor blood vessels into the interstitium. This is followed by travelling through the extracellular matrix (ECM) towards the tumor cells. In addition to the physical barriers posed by the endothelial cells, basement membrane and ECM, high interstitial pressure and low microvascular pressure may also interfere with extravasation of molecules and cells into tumor tissue (25).

Tumor blood vessel permeability is governed by the type of the tumor, the site of the vessels within the tumor, tumor growth rate, and tumor size. Permselectivity of tumor vessels is less than of normal vessels, presumably due to the large pores in the vessel wall. The transport of macromolecules, of which the permeability varied approximately two-fold within the molecular weight range of 25 kD to 160 kD, appeared to be limited by diffusion through these pores (28). However, most hyperpermeable blood vessels are located at the tumor-host interface. In addition, along the length of a single vessel significant differences were found (29). In studies using tracer amounts of monoclonal antibodies, extravasation of monoclonal antibodies took place mainly from these blood vessels located at the tumor-host interface and the vast majority of antibodies never actually made contact with the tumor cells (29).

In the case of cellular trafficking, movement of immune effector cells may also be impeded by the above mentioned characteristics of the tumor microenvironment (25). Furthermore, the requirement of adhesion molecules for the recruitment of immune effector cells presents another major obstacle in targeted cellular immunotherapy. The observation in an *in vivo* rat mammary adenocarcinoma skinfold chamber model that the interaction between leukocytes and endothelium in tumor microvessels was significantly diminished, was most likely a result of improper expression of adhesion molecules (30). Recently it was shown *in vitro* and *in vivo* that the angiogenic peptides VEGF and bFGF have different effects on the adhesion of a subpopulation of natural killer cells to endothelial cells. VEGF promoted adhesion, whereas bFGF inhibited adhesion. Both effects were mediated by differential expression of ICAM-1 and VCAM-1 by tumor vascular endothelium. Interestingly, the effect of VEGF on T lymphocyte adhesion to endothelial cells was completely absent (31). In *in vitro* experiments, Griffioen *et al.* have demonstrated an inhibitory effect of tumor derived angiogenic factors on inflammatory cytokine induced expression of endothelial cell adhesion molecules (32).

Immunohistochemical analysis of colorectal malignancies revealed a highly heterogeneous expression of E-selectin, VCAM-1 and ICAM-1 by tumor vascular endothelial cells (33). We have studied the expression of E-selectin, VCAM-1 and ICAM-1 by vascular endothelium of 20 human lung carcinomas and found no expression of E-selectin, VCAM-1 expression on less than 5% of the blood vessels and a slightly increased expression of ICAM-1 on a heterogeneous population of tumor blood vessels (unpublished observations). These and other studies indicate the existence of a tumor derived escape mechanism from the cytotoxic effector leukocytes via interference with the concerted expression of endothelial adhesion molecules.

Overcoming the Permeability Barrier

One way to manipulate the distribution of conventional therapeutics or drug delivery conjugates in tumor tissue is to increase vascular permeability of tumor vessels. This can be done by selectively targeting vasoactive compounds to tumor vasculature using carrier molecules directed against endothelium or a subendothelial component. The potential of this approach was shown by Epstein *et al.* using an anti-fibronectin directed antibody chemically conjugated with the vasoactive agent Interleukin-2. Pretreatment of tumor bearing mice with this conjugate 3 h before injection of a radiolabelled antibody directed against the tumor cells resulted in a three fold higher tumor uptake of the radiolabel (34). Furthermore, the physicochemical characteristics of the drug delivery macromolecules can be modified to create an increased transendothelial transport into the tumor tissue. In our laboratory, we have recently shown that mild chemical modification of proteins with certain anhydrides can induce an increased distribution of unchanged protein into the lymphatic system (35), confirming previous data of Lanken *et al.* (36). This effect probably reflects the normal transport route of plasma proteins across the endothelium (9). Such charge modifications may be exploited to selectively increase intratumoral disposition of anti-tumor vascular endothelium directed drug delivery preparations or other macromolecules. As the tumor levels achieved by passive accumulation seem related to plasma circulation time (8), changing the pharmacokinetics of a macromolecular carrier/drug conjugate may also improve distribution into the tumor.

A new trend in the development of antibody-antigen based recognition is the use of antibody fragments instead of whole immunoglobulin molecules. The molecular size decreases from 150 kD for an IgG via 50 kD of a Fab' fragment to 27 kD for a single chain Fv protein (ScFv). The greatest potential for use of ScFv is in antibody targeted therapy. Because they are prepared by molecular biology techniques they can readily be incorporated into fusion proteins with therapeutic entities. It was recently shown that anti-CEA located all known tumor deposits in patients with CEA-producing cancers (37). Tumor to blood ratios, important for tumor imaging, were up to 5.6:1 24 hrs after administration, which compares with ratios between 1 and 1.5:1 for whole IgG anti-CEA antibody. Tumor tissue accumulation was however slightly reduced compared to IgG. This is probably due to the fact that ScFvs are monovalent for antigen and are rapidly cleared from the general circulation. In the future increasing binding affinities of ScFvs may be obtained through affinity maturation. In addition, better tumor tissue accumulation may be achieved through manipulation of physicochemical and pharmacokinetic characteristics of the ScFv molecules.

Angiogenesis as a Target for Therapeutic Intervention

The awareness of the importance of angiogenesis in the growth of solid tumors led to a new concept for therapy of these tumors (38). An extensive search for the mechanisms underlying neovascularization in general and in tumors in specific resulted in the discovery of the tumor derived angiogenesis inhibitors thrombospondin and angiostatin. These agents not only inhibit new blood vessel formation and thereby limit tumor tissue perfusion, but probably also interfere with the paracrine production of endothelial growth factors that stimulate tumor cells (39). Angiostatin inhibited growth of metastases and primary tumors in a murine Lewis Lung carcinoma model (40) as well as in a murine fibrosarcoma (39). A fumagillin analogue, AGM-1470 (TNP-470), inhibited *in vitro* endothelial cell proliferation and migration at concentrations that did not affect other cell types. Furthermore, TNP-470 was shown to be highly effective in the treatment of experimental tumors with no evidence of drug related toxicity (41).

Antibodies directed against VEGF peptides were capable of blocking VEGF permeability activity and VEGF mediated endothelial cell growth *in vitro* (23). *In vivo*, anti-VEGF antibodies were active in inhibiting tumor growth and metastases in a number of experimental tumor models (42,43).

Epitopes on Tumor Vascular Endothelium for Selective Targeting

Tumor endothelial cells are a suitable target for drug delivery strategies and immunotherapy as they are freely accessible through the blood. Furthermore, approaches directed against tumor endothelium may be applicable to most or possibly all solid tumors as all tumors rely on similar types of blood vessels for growth (44). It should be kept in mind, though, that due to the heterogeneity in angiogenic peptide expression and other tumor growth related characteristics, a homogeneous endothelial cell population is unlikely to be found within solid tumors.

One of the first studies on the development of molecules specifically recognizing tumor endothelium (Table 1) was published by Hagemeier *et al.* in 1986 (45). They developed an antibody (EN 7/44) recognizing a 30.5 kD antigen mainly present in proliferating tissue (placenta, umbilical vein, intestine) and in acute inflammatory reactions and tumors. The antibody recognized endothelial cells at the tip of budding capillaries. Immunohistochemal staining of tumor tissue with anti-CD34 antibodies also identified an angiogenesis related endothelial epitope present on abluminal processes mostly at the tips of vascular sprouts (46). The restricted distribution of endosialin, a 165 kD cell surface glycoprotein, by the vascular endothelial cells of malignant tumors makes this antigen a potential target for tumor vascular targeting (47). In 67% of the 128 tumor samples tested by immunohistochemistry the endosialin epitope could be detected. Normal blood vessels and other adult tissue lacked expression. The rapid internalization of anti-endosialin monoclonal antibodies (MoAbs) by the target cells offers the possibility of using these antibodies for intracellular delivery of cytotoxic or other modulatory agents.

In a study of *in vivo* distribution of anti-VEGF polyclonal antibodies in tumor bearing mice, maximal accumulation of antibodies in two well-vascularized tumors was 16% and 13% of injected dose per gram tissue (48). Lesser accumulation was found in poorly vascularized tumors (7% I.D./g tissue).

Table 1. Potential Target Epitopes on Tumor Vascular Endothelial Cells, Basement Membrane or Stromal Components

Target epitope	Location of the target epitope	Ref.
30.5 kD antigen	proliferating tissue, acute inflammatory reactions, tumors	Hagemeijer (45)
CD34	tips of vascular sprouts	Schlingemann (46)
Endosialin	vascular endothelial cells of malignant tumors	Rettig (47)
VEGF/VEGF-R complex	VEGF-R overexpressed by endothelial cells in many tumors	Brown (50)
Endoglin (TGFβ receptor complex component)	endothelial cells in miscellaneous human tumors (?)	Burrows (53)
F19 cell surface glycoprotein	stromal fibroblasts in stroma of > 90% of epithelial cancers	Garin-Chesa (55)
Fibronectin	basement membrane component	Epstein (34)
Fibrin	stroma component	Kairemo (56)

Accumulation in normal tissue was always less than 5% I.D./g tissue. In comparison, antibodies directed against tumor cells accumulate to a much lesser extent, varying from 0.0001 to 0.01% of the injected dose per gram tumor tissue (49). As VEGF and VEGF receptor (VEGF-R) expression are significantly increased in many different tumor types (50,51), VEGF or the VEGF/VEGF-R complex on tumor vascular endothelium may be a potential target for tumor vascular targeting. By now, it is uncertain whether the localization of VEGF/VEGF-R on the abluminal side and in the vesiculovacuolar organelles of the endothelium (52) will be an obstacle in exploiting VEGF or the complex as a target. Furthermore, localization of anti-VEGF antibodies or fragments in normal tissues producing VEGF could diminish tumor endothelium specificity. In this respect, the observation that in anti-angiogenesis studies with anti-VEGF antibodies no obvious toxicities were observed is an important finding (42). Lack of toxicity is probably a result of the fact that the VEGF/VEGF-R target on tumor vascular endothelium is more accessible for the antibodies than in normal tissues. As discussed above, this can be due to enhanced vascular permeability and/or the presence of an incongruous basement membrane in tumor vasculature. Another factor of importance for future therapeutic efficacy using anti-VEGF antibodies or fragments as carrier is the heterogeneity of VEGF expression within a solid tumor. Although we could demonstrate homogeneous expression of VEGF in s.c. solid squamous cell carcinoma in the rat, the expression in pulmonary metastases of the same type in the rat, and in human lung carcinomas was highly variable (unpublished observations).

Endoglin, an essential component of the TGFβ receptor complex of human endothelial cells was put forward as a proliferation marker that is upregulated on endothelial cells in miscellaneous human solid tumors (53). In another study, however, flow cytometry analysis of endoglin expression on ovarian carcinoma derived endothelium demonstrated a decrease of

endoglin expression to a level of 20% of normal endothelium (54).

Not only the tumor vascular endothelium is a target for the development of new, non-tumor cell directed drug delivery systems. Monoclonal antibodies directed against specific molecules in the basement membrane and subendothelial stroma of tumors have been developed. In combination with the enhanced leakiness of part of the tumor blood vessels, improved accumulation and selectivity of drug delivery systems may be created. One such target epitope is the F19 cell surface glycoprotein of reactive stromal fibroblasts present in the stroma of more than 90 percent of common epithelial cancers (55). In mouse tumor models, antibodies against the basement membrane component fibronectin were shown to be capable of delivering vasoactive agents to tumor tissue (34). Basement membrane components as target, however, may be hampered by the lack of basement membrane in a wide variety of tumors. Antibodies against fibrin were developed based on the observation that especially in tumor tissue fibrin deposition was a prerequisite for stroma deposition and extracellular matrix formation. In nude mice bearing human ovarian carcinoma xenografts tumor uptake of ^{90}Y-labeled anti-fibrin antibody was approximately 10% (I.D./g tissue) after intravenous injection (56).

Tumor Vasculature Directed Strategies in Cancer Research

As discussed above, tumor vascular endothelium may be a target for selective delivery of vasoactive compounds that will facilitate distribution of subsequently administered agents into the tumor tissue.

In the case of immunotherapy, one of the main obstacles for effective anti-tumor response may be the hampered expression of adhesion molecules by tumor vascular endothelium. Selective delivery of cytokines capable of inducing adhesion molecules may create circumstances that facilitate immune cell adhesion and transendothelial migration at the site of the tumor only. Using intravital microscopy of animal tumors, it was shown that TNFα can enhance leukocyte rolling and adhesion to tumor blood vessels. The response was dependent on tumor type, site of tumor growth and animal strain (57). As this technique is not (yet) capable of monitoring leukocyte diapedesis, the consequences of enhanced rolling and adhesion on transendothelial migration was not investigated. In a study by Renard *et al.* immune cell influx could be demonstrated in conjunction with induced adhesion molecule expression by tumor vascular endothelium (58). TNFα administered via extracorporeal isolated limb perfusion to patients with melanoma and sarcoma located on the lower limbs induced expression of ELAM-1, VCAM-1 and ICAM-1 on intratumoral endothelial cells. Significant influx into the tumor of polymorphonuclear cells, followed by T and B lymphocytes was observed during the first days to weeks after treatment (58).

One approach in immunotherapy is circumventing the lack of MHC restricted antigen recognition by immune effector cells using bispecific antibodies (Figure 3) (59). By cross linking effector cells (for example cytotoxic T lymphocytes (CTLs)) and tumor cells, the CTLs are capable of lysing the tumor cells *in vitro* and *in vivo* (60). In carcinoma patients, the combination treatment of s.c. IL-2 and i.v. BIS-1 F(ab')$_2$ bispecific antibody directed against epithelial glycoprotein-2 (EGP-2) and the

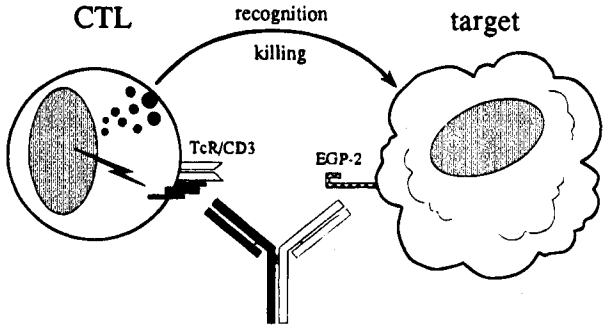

Fig. 3. Schematic representation of bispecific antibody mediated lysis of target cells. Through cross linking of the T cell receptor (TcR)/CD3 complex on the cytotoxic T lymphocyte and epithelial glycoprotein-2 (EGP-2) on the target cell, the lymphocyte is capable of actively lysing the target cell (from B. J. Kroesen, thesis University Groningen, the Netherlands, 1995, page 171).

TcR/CD3 complex on T lymphocytes elicited an immune response reflected by elevated plasma levels of TNFα and IFNγ (61). The accumulation of bispecific antibody loaded CTLs seems nevertheless not sufficient to create an effective anti-tumor response. To improve anti-tumor effectivity, we are now developing strategies that will selectively deliver CTLs at the tumor endothelium, followed by site specific activation leading to transendothelial migration and bispecific antibody mediated anti-tumor activity. Tumor growth can also be inhibited by interfering with tumor blood flow. This can be done either by damaging the endothelial lining resulting in the formation of thrombi or by directly manipulating coagulant activity (20). In an animal model of tumor vascular targeting, Burrows and Thorpe demonstrated that endothelial damage provoked by an anti-tumor endothelium directed ricin A chain-immunotoxin resulted in dramatic regressions of large solid tumors (62). The immunotoxin could not cure the animals because a small population of tumor cells at the tumor-host interface survived the treatment. A combination therapy of anti-tumor endothelium and anti-tumor cell directed immunotoxins induced permanent complete remissions in over half of the animals.

Due to the toxic side effects of ricin A and the aim to completely humanize active proteins, another approach in the same animal model was developed based on site specific coagulation (63). Bispecific antibodies directed against tumor endothelium on one hand and tissue factor (the initiator of the extrinsic pathway of blood coagulation) on the other hand, were constructed. *In vivo* application of the bispecific antibody plus a truncated form of tissue factor directly induced thrombotic occlusion of the tumor blood vessels without signs of toxicity (63,64). Yet another option to cause blood vessel occlusion is through direct lysis of endothelial cells by immune effector cells. This may be achieved via cross linking of CTLs (or other effector cells) and tumor vascular endothelium using bispecific antibodies directed against specific epitopes on both cell types. Understanding the intracellular mechanisms by which growth of angiogenic vessels in the tumor is regulated may provide tools for interventions at this level using targeting moieties that are internalized by the endothelial cells. For example, tumor regression can be promoted via integrin $\alpha_v\beta_3$ by inducing apoptosis of angiogenic blood vessels (65). Manipulation of the regulatory pathway causing apoptosis through e.g. gene

targeting may be a future approach. Also, genes that are directly cytotoxic to the endothelial cells or that activate toxic prodrugs may have a significant impact on tumor growth (22). The inclusion of endothelial cell specific promotors and enhancers may improve selectivity and expression (66).

Specific antisense oligomers complementary to angiogenic peptides like bFGF and TGFβ1 have been shown to effectively block their synthesis by confluent, quiescent smooth muscle cells and may also be useful in the manipulation of expression of angiogenesis factors in endothelial cells (22).

Dual Targeting

Future therapeutic drug delivery strategies will consist of combination treatments. No strategy will have a future on its own. As the tumor can escape via numerous (some unidentified) routes, an approach aimed at one process, mechanism or epitope is doomed to fail.

In our laboratory, we have developed (glyco)protein carriers with intrinsic antiviral, anti-inflammatory or anti-endotoxin activities (67). Conjugation of these carriers with drugs results in preparations that have 'dual targeting' potential (68). With regard to dual targeting strategies for anti-tumor therapy, we are now investigating the possibilities of interfering with angiogenesis on one hand and creating an effective immune response against the tumor on the other. This may result in a synergistic effect as it can interfere with independent, unrelated processes in tumor growth.

Antibodies or antibody fragments directed against VEGF as targeting/carrier moieties for pharmacologically active agents are interesting candidates for use in dual targeting approaches. They have a significant anti-tumor effect *in vivo* in tumor bearing animals after intravenous administration (42). Bispecific antibodies against VEGF and TNFα or anti-VEGF ScFv-TNFα fusion proteins, may combine the anti-angiogenesis effect of the anti-VEGF antibody with the immune effector cell recruitment potential of TNFα.

SUMMARY AND CONCLUSIONS

The blood vessel organization within solid tumors forms a significant obstacle in the therapy of these malignancies. Vascular permeability is highly heterogeneous, as is the expression of adhesion molecules. This leads to irregular disposition of conventional chemotherapeutic drugs, advanced drug delivery preparations and immunocompetent cells to the tumor cells.

A potential approach to overcome the problems that therapeutics encounter en route to their target cells, is to develop therapeutic entities aimed at the tumor vasculature. These entities can either create more homogenously distributed enhanced vascular permeability, inhibition of tumor blood flow or increased immune effector cell influx.

The tumor vasculature specific epitopes that have been described in this paper are factors endogenously produced/expressed by tumor cells or tumor tissue associated cells and components. Although not homogenously distributed within the tumor tissue, they will offer the opportunity to investigate new therapeutic strategies in *in vivo* experimental models, while the search for other target epitopes continues.

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