Biochemical Pharmacology, Vol. 55, pp. 1939–1945, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00011-2

COMMENTARY

Tumor Vasculature Targeted Therapies

GETTING THE PLAYERS ORGANIZED

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ABSTRACT. Based on their location and central role in solid tumor growth, tumor vascular endothelial cells may present an attractive target for the delivery of therapeutic drugs or cells. The potency of blocking the tumor blood supply in eradicating solid tumors was demonstrated recently in a mouse model of tumor vasculature targeting (Huang *et al.*, *Science* **275:** 547–550, 1997). For clinical application of such strategies, tumor endothelium specific target epitopes need to be identified. Recent studies on angiogenesis have identified angiogenesis-related molecules as potential target epitopes. Among these are vascular endothelial growth factor (VEGF)/VEGF-receptor complex, αv integrins, and Tie receptor tyrosine kinases. Besides blockade of their signalling cascades leading to inhibition of angiogenesis, these epitopes may also be instrumental in tumor vessel specific delivery of therapeutics. Data on the efficacy of therapeutic modalities aimed at these, mostly heterogeneously distributed tumor endothelial epitopes are scarce, and sophisticated experimentation is required to rationalize the development of new therapeutic strategies. Importantly, only detailed evaluations in cancer patients will provide the blueprint for the development of clinically effective tumor vascular targeted therapies. BIOCHEM PHARMACOL **55**;12:1939–1945, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. tumor; vasculature; targeting; target epitopes; animal; human

The treatment of solid tumors using chemotherapeutic agents is a difficult task due to a number of factors: limited accessibility of tumor tissue, occurrence of multidrug resistance, major/intolerable toxicity of the anti-cancer drugs and heterogeneity of tumor tissue. To circumvent toxicity and/or to increase the effectiveness of anti-neoplastic therapy, approaches have been developed that aim at selectively delivering the pharmacologically active compound to the tumor or attempt to target enzymes that can activate prodrugs at the tumor site (for reviews see Refs. 1–5). The success of these so called "drug-targeting" strategies lies in the selectivity of the interaction between the carrier molecule and its target epitope. Furthermore, as with any other therapy, accessibility of the target tissue for the carrier-drug conjugates or prodrug-activating modality is of fundamental importance.

By now, it has become increasingly clear that this last feature in particular impedes the effectiveness of drug-targeting strategies *in vivo*. Using immunotoxins (conjugates of antibodies/antibody fragments and plant or bacterial toxins) for example, encouraging responses have been observed in patients with hematological malignancies. Treatment of solid tumors, however, has not been successful due to (among other factors) poor penetration of the conjugates in solid tumor masses [6]. It is widely accepted, therefore, that treatment with immunotoxins should concentrate on patients with minimal residual disease. Yet, the quest for effective procedures for the treatment of solid tumor masses continues.

TUMOR VASCULAR ENDOTHELIUM AS A TARGET

The vasculature in solid tumor tissue is highly disordered, with numerous vascular shunts, irregular vascular diameters, wide interendothelial junctions, large numbers of fenestrae and transendothelial channels, and discontinuous or absent basement membranes [7, 8]. Although overall vascular permeability of tumor blood vessels is increased as compared with normal tissue vessels, not all tumor blood vessels are leaky. In addition, the tumor blood vessel walls and high interstitial fluid pressure within the tumor tissue form a major barrier for the transport of tumor cell-directed therapeutic modalities [1, 9]. Based on their location, the cells lining the tumor blood vessel wall present a much more attractive site for specific delivery of therapeutics than tumor cells, as they are more easily accessible from the blood. Furthermore, tumor endothelial cells themselves may be a lucrative target for therapeutic intervention since most tumor cells rely for their growth and survival on an intact blood supply. While tumor cells are genetically unstable, rapidly mutating, and able to develop multidrug

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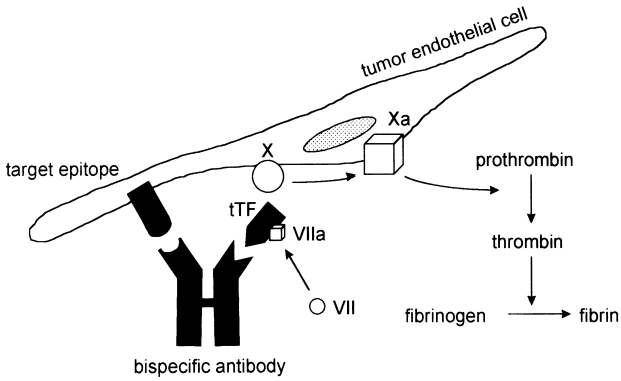
resistance, vascular endothelial cells are genetically stable and rarely become drug resistant [10].

COAGULIGAND-INDUCED TUMOR BLOOD FLOW BLOCKADE

In the 1970s and early 1980s, Dr. J. Folkman and Dr. J. Denekamp pointed to the importance of the tumor blood supply for solid tumor growth and the potential for attacking it as a means of anti-tumor therapy [11, 12]. Recently, one of us (G. M.) showed that blockade of the blood supply of a solid tumor indeed caused a dramatic reduction in tumor mass [13]. Through local delivery of a coagulation factor to the tumor blood vessel wall, a rapid induction of blood coagulation was created, leading to extensive tumor cell death.

Imperative for the success of the approach was the selectivity of the delivery of the coagulation factor at the site of the tumor vascular endothelium. To achieve such selectivity, we utilized a mouse model for tumor vasculature targeting developed by Burrows *et al.* [14]. In this model, subcutaneously inoculated neuroblastoma tumor cells transfected to produce IFNγ* induced a local up-regulation of MHC Class II on the tumor vascular endothelial cells. Antibodies directed against these MHC Class II molecules homed selectively to the tumor vasculature after intravenous administration. Although immunotoxins derived from MHC Class II directed antibodies effectively killed the tumor endothelial cells [15], toxicities related to the immunotoxins led the research team to investigate a more refined method for interfering with the tumor blood supply. For this purpose, the extraordinary capacity of TF to initiate the blood coagulation cascade through sequential activation of a series of coagulation factors was employed [16]. The use of a truncated form of TF (tTF; [17]) was essential in this approach. In contrast to full-length TF, which is highly toxic when administered systemically due to the rapid induction of disseminated intravascular coagulation, tTF is devoid of coagulation induction activity. Only if it is brought into close contact with a membranous structure containing factor X will it regain its activity to induce blood coagulation. Systemic administration of microgram amounts of tTF was not toxic for mice. However, bringing together tTF and factor X via targeted cross-linking of tTF to the membranes of target cells restored coagulation induction activity. tTF and target cell cross-linking was done by a bispecific antibody combining MHC Class II specificity with TF specificity (Fig. 1). *In vivo*, this site-specific blood coagulation induction in the tumor blood vessels resulted in blockade of the tumor blood flow and



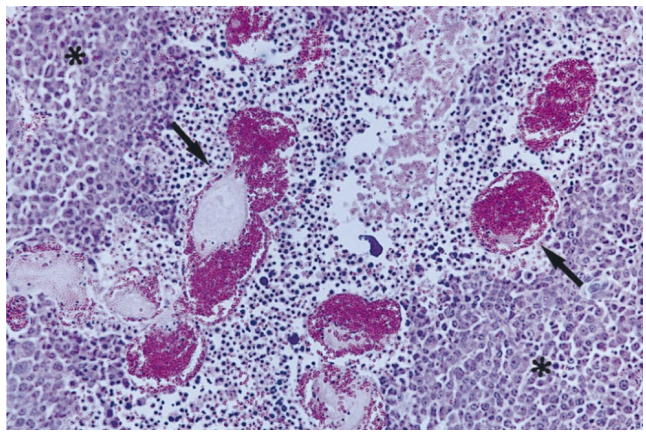


FIG. 1. Top panel: Schematic representation of the putative mechanism of coaguligand-induced blood coagulation. The coaguligand (MHC Class II × TF directed bispecific antibody complexed with tTF) selectively binds to the target epitope on the tumor endothelial cells. Bringing the tTF/factor VIIa complex close to the target cell membrane allows an interaction between tTF/VIIa and factor X, thereby initiating the coagulation cascade. Bottom panel: Hematoxylin/eosin staining of mouse tumor tissue 24 hr after a single dose of bispecific antibody · tTF complex. Erythrocytes (dark pink) are trapped in the blood vessels (arrows) by fibrin clots (pink). The blockade of tumor blood flow resulted in advanced tumor cell necrosis alongside the coagulated vessels. Those tumor cells not deprived of blood supply are still viable (asterisks). The blood vessels in the latter areas lacked expression of the target antigen.

massive tumor cell death. Nearly 40% of mice bearing large s.c. tumors could be cured after two i.v. injections of bispecific antibody · tTF coaguligand complex. In mice that did not show complete regressions, immunohistochemical analysis revealed that the vascular endothelium lacked MHC Class II target antigens, explaining the lack of thrombosis induction in these vessels [13].

CLINICALLY RELEVANT TUMOR VASCULATURE SPECIFIC EPITOPES

In the coaguligand approach in the mouse model, the selectivity of coagulation induction at the tumor vasculature was created by the (artificially induced) specific expression of MHC Class II molecules in the tumor vasculature. MHC class II is not likely to be a target epitope of

* *Abbreviations:* bFGF, basic fibroblast growth factor; BsMAb, bispecific antibody; CTL, cytotoxic T lymphocyte; FAA, flavone acetic acid; IFNγ, γ-interferon; MoAb, monoclonal antibody; PD-ECGF, platelet derived-endothelial cell growth factor; RTK, receptor tyrosine kinase; TcR, T cell receptor; TF, tissue factor; TNFα, tumor necrosis factor-α; tTF, truncated tissue factor; VEGF (−R), vascular endothelial growth factor (− receptor).

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 kDa antigen	Endothelial cells in proliferating tissue, acute inflammatory reactions, tumor	[18]
CD34	Tips of vascular sprouts	[19]
Endosialin	Endothelial cells of malignant tumors	[20]
Endoglin	Endothelial cells in miscellaneous human tumors	[21]
CD44	Activated/angiogenic human endothelial cells	[22]
F19 cell surface glycoprotein	Stromal fibroblasts in >90% of epithelial cancers	[23]
Fibronectin	Basement membrane component	[24]
Fibrin	Stromal component	[25]

choice in the clinic. In the search for tumor vascular specific markers for diagnostic use as well as for future tumor vasculature targeted therapies, a number of epitopes have been proposed in the last decade as being tumor blood vessel specific (Table 1). In addition to markers specific for the endothelial cells, stromal components have also been put forward for use in tumor targeting. The reason for this is that they may be accessible from the blood due to increased permeability of the tumor vasculature. Most of the putative target epitopes in Table 1 have been reviewed recently in greater detail by us and will not be discussed further [1].

At the moment, a number of exciting developments are in progress aiming at more recently discovered tumor vasculature specific epitopes. As these epitopes are, in one way or another, involved in angiogenesis-related processes, we will briefly discuss some events that are of importance during angiogenesis. For more detailed information on angiogenesis and the regulation of angiogenesis, see Ref. 26.

ANGIOGENESIS AND TUMOR GROWTH

Angiogenesis, the formation of capillaries from pre-existing blood vessels, is of fundamental importance in physiological processes (e.g. embryogenesis, wound healing), and in pathological conditions such as diabetic retinopathy and tumor growth. Tumor nodules of 1–2 mm in diameter can grow by deriving nutrients via diffusion. For additional growth, neovascularization is obligatory to provide an adequate blood supply and is an important step in the progression of a tumor [27]. One of the most striking observations in tumor blood vessel research is that tumor endothelium proliferates 20–2000 times faster than any normal (except placental) tissue endothelium in the adult [12]. Hypoxia, and probably also a variety of other still uncharacterized stimuli, induces various cells (monocytes/macrophages, infiltrating lymphocytes, connective tissue cells, endothelial cells, tumor cells) to produce angiogenic peptides. At the same time, anti-angiogenic peptides can be generated. The net balance of pro- and anti-angiogenic factors will determine whether new blood vessels are formed or not [28].

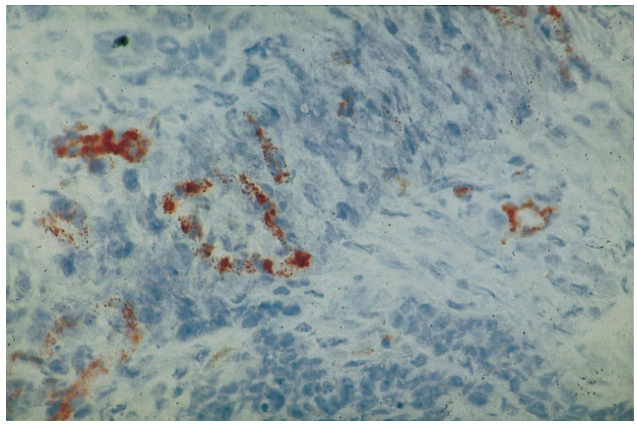


FIG. 2. Immunohistochemical analysis of rat tumor tissue grown subcutaneously using MoAb 3H12 directed against VEGF/VEGF-R complex. The majority of tumor blood vessels stained in a granular staining pattern formerly described for VEGF-R distribution [35]. Blood vessels in the liver and kidney did not react with the antibody, whereas in the lungs staining was observed in restricted vascular sites (data not shown).

The initiation phase of angiogenesis is marked by activation of endothelial cells through mediators such as bFGF, VEGF, PD-ECGF, TNFα and ligands for the receptor tyrosine kinases Tie1 and Tie2 [29, 30]. Activated endothelial cells proliferate and exhibit an elevated expression of cell adhesion molecules and proteolytic enzymes. Cooperation between adhesion molecules such as integrins and proteolytic enzymes is an important prerequisite for sprouting blood vessels to invade [31, 32]. In addition to integrins being involved in blood vessel maturation [29], the endothelial specific receptor tyrosine kinase Tie1 is functionally important for endothelial cell differentiation and establishment of blood vessel integrity [30].

VEGF/VEGF-RECEPTOR COMPLEX AS A TARGET

VEGF (also known as vascular permeability factor) is an angiogenic growth factor produced at high levels by a large number of solid tumors. Neutralizing MoAbs directed against VEGF were shown to block the angiogenic activity of VEGF, resulting in significant permeability changes, tumor vascular regression [33], and anti-tumor effects [34].

For tumor vascular targeting purposes, we recently developed MoAbs directed against the complex of VEGF and its receptors (Fig. 2). In addition to an increased expression of VEGF by tumor cells, VEGF-R expression is up-regulated significantly in tumor endothelial cells (for reviews on the physiology and pathophysiology of VEGF and its receptors, see Ferrara and Keyt [36] and Brown *et al.* [37]). We hypothesized that an antibody directed against the VEGF/VEGF-R complex might provide an opportunity to develop so-called "dual-targeting" strategies [2]. In these "dual-targeting" strategies, the therapeutic entity consists of a

carrier molecule, exerting a pharmacologic activity itself, combined with an active drug, protein or cell. It is anticipated that the binding of an antibody to VEGF/VEGF-R complex on tumor vascular endothelium may block the signalling cascade as well as provide a tool for the selective delivery of, for example, TNFα, tTF, or immunocompetent cells. Experiments to study the potential of anti-VEGF/VEGF-R complex antibodies for selective tumor blood vessel targeting and use in "dual-targeting" strategies are in progress.

αVβ3 INTEGRIN AS A TARGET

αvβ3 Integrin is a marker in angiogenic endothelium [32], being expressed on the apical surface of the blood vessels [38]. Preventing the αvβ3 integrin from binding to its ligand results in apoptosis of endothelial cells of newly formed blood vessels [39]. Peptides mimicking ligands of the αvβ3 integrins and antibodies capable of inhibiting ligand–integrin binding exhibit anti-angiogenic and anti-tumor effects [39, 40].

By intravenous injection of phages displaying RGD-containing peptides with high affinity for the αvβ3 integrins into tumor-bearing mice, Pasqualini *et al.* [41] recently showed that αvβ3 integrins are a potential target for use in tumor vasculature targeting. The nonapeptide expressed by the phages contained an RGD sequence in a cyclic conformation with two disulfide bonds, which is highly selective for the αv integrins. Whereas the RGD peptide itself distributed and bound equally well to tumor cells as to tumor endothelium, the phages expressing the RGD-peptide only targeted the endothelium. This difference is due most likely to the size of the phage, which is unlikely to penetrate into tissues.

The RGD-containing cyclic peptides may themselves exhibit anti-angiogenic properties through induction of endothelial cell apoptosis [39]. Linking these peptides to, for example, tTF, angiostatin [42], or other pharmacologically active molecules may concentrate these molecules at the tumor vessels and, hence, increase their therapeutic efficacy [10]. As with antibodies against VEGF/VEGF-R complex, dual activities of such conjugates may be envisioned.

TIE RECEPTORS AS A TARGET

Tie1 and Tie2 define a class of RTKs that are primarily expressed in developing vascular endothelial cells. While the function of Tie1 is related to endothelial cell differentiation and the establishment of blood vessel integrity, Tie2 is involved in angiogenic processes [30].

A naturally occurring ligand that signals through Tie2 RTK is angiopoietin-1 [43]. Recently, a factor closely related to angiopoietin-1, termed angiopoietin-2, was identified. This latter factor is a naturally occurring antagonist for angiopoietin-1 and its Tie2 receptor [44]. The roles of the angiopoietins and the RTKs in physiological and pathological angiogenesis need to be further established. It may, however, be clear that those protein sequences of angiopoietin-2 responsible for blockade of Tie2 RTK may be exploited for tumor vasculature targeting purposes in the future.

TARGETED THERAPEUTIC STRATEGIES

Besides the selective delivery of a blood coagulation-inducing factor like TF, other strategies can be exploited for interfering with the blood supply of solid tumors. As already mentioned, intervention at the level of angiogenesis may lead to significant tumor mass reduction. This has been shown for antibodies neutralizing VEGF activity and interfering with αvβ3 integrin-ligand binding. Thus far, no drug resistance has been observed in animal experiments and clinical trials during long-term anti-angiogenic therapy directed against the endothelial cell population of a tumor [10].

Direct cytotoxicity against tumor vascular endothelial cells may be induced using hybrid molecules able to functionally cross-link immune effector cells and tumor endothelial cells [3]. For this purpose, BsMAbs recognizing both tumor vascular specific epitopes and CTLs as effector cells are under development in our laboratories. CTLs were shown to be highly effective in killing target cells when cross-linked by CD3 × tumor-associated antigen-directed BsMAbs *in vitro* and *in vivo* [45, 46]. One potential advantage of such an approach may be the possibility to create an inflammatory response at the site of the tumor upon CTL activation and endothelial cell killing. Blockade of the tumor blood supply and induction of inflammatory reactions may act synergistically in reducing the tumor load.

Growth-inhibiting substances may be delivered at the tumor vascular endothelial cells as well. From prior experience in drug delivery research, one would expect an optimal effect (i.e. highest activity and least toxicity elsewhere in the body) of these compounds when delivered intracellularly. No data are available yet to answer the question whether antibodies directed against the tumor endothelial epitopes discussed above are capable of inducing endocytosis, e.g. via receptor clustering. On the other hand, tumor endothelial cells may be quite sensitive to toxic compounds due to their high proliferation rate. Possibly, local gradual release of therapeutic agents at the tumor endothelium may be sufficient as well to induce significant endothelial cell killing without exhibiting systemic toxicity.

TARGET EPITOPE CONSIDERATIONS

With respect to the choice of the target epitope to which therapy is directed, a number of important issues should be considered.

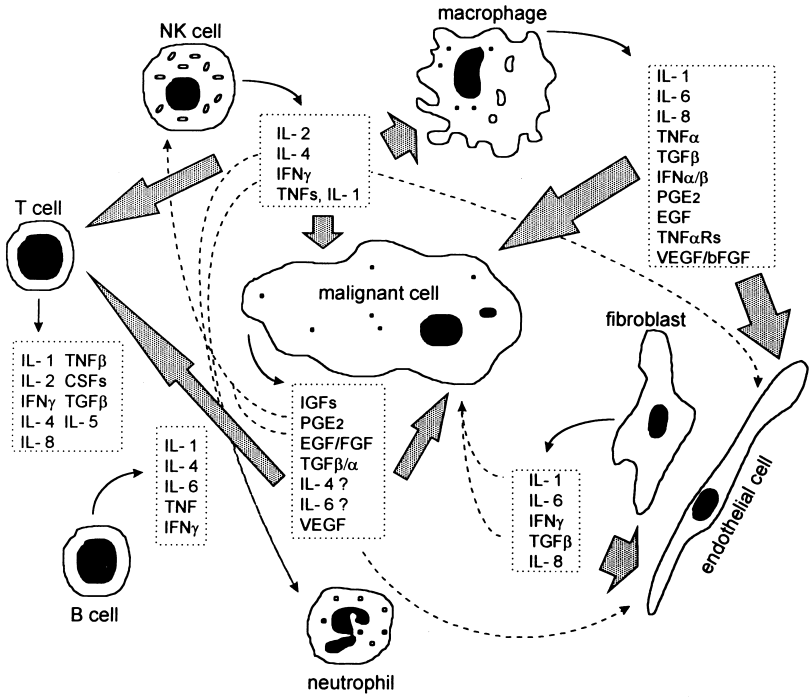


FIG. 3. Schematic representation of the cytokine network that may exist in solid tumors. A variety of cytokines are produced by tumor cells, endothelial cells, fibroblasts, and infiltrating immune cells that stimulate or inhibit the activity of neighbouring cells (adapted from Ref. 48).

Target Epitope Distribution within the Tumor

One important issue is the distribution of the selected target epitope on the tumor vascular endothelial cells. In mice not responding or partially responding to coaguligand therapy, the target epitope was shown to be absent from the vessels [13]. Although growing solid tumors do exhibit an angiogenic phenotype, not all parts of the tumor blood vessels will be actively participating in angiogenesis at the same time. Immunohistochemical analysis of human lung carcinoma biopsies indeed revealed a heterogeneous VEGF distribution (unpublished observations). A similar heterogeneity in expression was seen with the angiogenic factor bFGF [47]. Likely, the complex network of cytokines and angiogenic factors produced in a tumor (Fig. 3) results in different levels of expression of target epitopes within one tumor, and between tumor types [48].

Selectivity of the Target Epitope for Tumor Vasculature

VEGF, $\alpha v\beta 3$ integrins, and Tie RTKs have a role in normal vascular modelling. It is therefore conceivable that interference at this level may also have an effect on physiological homeostasis, leading to unwanted side-effects. The lack of toxicity in combination with effective tumor growth inhibition after treatment of animals with VEGF neutralizing antibodies [34] or angiostatin [49] are, however, quite encouraging.

Density of the Target Epitope

A minimum of tTF molecules per target cell is required for initiating blood coagulation after cross-linking at a target cell. *In vitro*, we observed a significant induction of blood coagulation when 300,000 tTF molecules were cross-linked per target cell [13]. Even 20,000 tTF molecules per target cell accelerated blood coagulation *in vitro*. No data are available, however, on the number of tTF molecules delivered in the *in vivo* situation to the vasculature of the coaguligand-treated mice responding with rapid blood coagulation.

The threshold for activating CTLs through triggering of the TcR was estimated to be ~8000 TcR per cell, irrespective of the nature of the triggering ligand. Costimulatory signals lowered this activation threshold to ~1500 TcR [50]. For subsequent engagement of cytotoxic T lymphocytes in, for example, BsMAb-directed target cell killing, the actual number of required attachment sites is unknown. From *in vitro* data on the relation of BsMAb concentration and killing activity, it was calculated that cytolytic activity can be observed when CD3/TcR occupancy is as low as 200 per CTL.

In various cultured endothelial cells, VEGF-Rs flt-1 and KDR/flk-1 are present at frequencies of ~3000 and 40,000 copies per cell, respectively (see Ref. 37 for original papers). On the other hand, bovine retinal endothelial cells had a single class of high-affinity receptors present at a density of 100,000 per cell [51]. Based on the *in vitro* coagulation data discussed above, VEGF/VEGF-R targeted tTF may accommodate blood coagulation *in vivo*. The same holds true for VEGF/VEGF-R directed cross-linking of CTLs and tumor endothelium. Nevertheless, only *in vivo* evaluation of the delivery of pharmacologically active agents or cells at the site of tumor endothelial cells in tumor-bearing animals and cancer patients will provide insight in the potentials of these approaches for future clinical use.

OF MICE AND MEN

Extrapolation of studies performed in experimental animals to clinical situations is complex. One important aspect that should be considered here is the existence of significant intra- and inter-species variations in the tumor cell and tumor blood vessel microenvironment. It has been observed that monoclonal antibodies distribute to a much greater extent into animal tumors than into tumor tissue of patients. This difference may be explained by differences in vascular permeability and intratumoral pressure between human and animal tumors. Moreover, differences between different types of human tumor xenografts in mice have been observed with respect to vascular resistance and interstitial pressure [9, 52].

Experience with the synthetic flavonoid FAA in anti-tumor therapy presented evidence that human tumor vasculature may respond differently to vasculature-directed stimuli than mouse tumor vasculature. FAA exerts its anti-tumor activity via TNFα-mediated induction of hemorrhagic necrosis [53]. In addition, tumor endothelium may be a critical target for FAA activity via stimulation of the nitric oxide pathway [54]. In mice bearing solid tumors, FAA exhibited impressive anti-tumor activity. This is in sharp contrast with the effects seen in the clinic, where administration to patients with progressing metastatic melanoma had no effect whatsoever [55].

It is especially important in the development of therapies based on new concepts that great care be taken in the extrapolation of animal data to the human situation. More information on, for example, target epitope density and distribution, and coagulation status in experimental animals and cancer patients is required. Until this information is available, proper interpretations regarding potentials and limitations of tumor vasculature targeted therapies will be difficult to make.

CONCLUSIONS

Tumor vasculature targeted therapies hold great promise for the treatment of solid tumors in the future. New molecules, most of which are angiogenesis related, are regarded as potential target epitopes. Important characteristics of target epitope expression (heterogeneity, normal physiology, and frequency of expression in animal and human tumors) should be taken into account for the development of effective therapeutic strategies. Probably no single strategy on its own will be successful in eradicating solid tumors in cancer patients. Through collaborative efforts, the different strategies should therefore be combined into a winning team. Although some of the players have now been identified, their position and function within the team need to be established.

The authors wish to acknowledge the "Digitaal Centrum" of the Faculty of Medicine and the University Center for Pharmacy, University of Groningen, and "Foto Jos Lange, Groningen" for processing of photographic material.

References

1. Molema G, de Leij LFMH and Meijer DKF, Tumor vascular endothelium: Barrier or target in tumor directed drug delivery and immunotherapy. *Pharm Res* **14:** 2–10, 1997.
2. Meijer DKF, Molema G, Moolenaar F, De Zeeuw D and Swart PJ, (Glyco)-protein drug carriers with an intrinsic therapeutic activity: The concept of dual targeting. *J Controlled Release* **39:** 163–172, 1996.
3. Kroesen BJ, Helfrich W, Molema G and de Leij L, Bispecific antibodies for the treatment of cancer in experimental animal models and man. *Adv Drug Delivery Rev*, in press.
4. Meijer DKF and Molema G, Targeting of drugs to the liver. *Semin Liver Dis* **15:** 202–256, 1995.
5. Senter PD, Activation of prodrugs by antibody–enzyme conjugates: A new approach to cancer therapy. *FASEB J* **4:** 188–193, 1990.
6. Frankel A, Fitzgerald D, Siegall C and Press O, Advances in immunotoxin biology and therapy: A summary of the Fourth International Symposium on Immunotoxins (Myrtle Beach, South Carolina, USA, June 8–10, 1995). *Cancer Res* **56:** 926–932, 1996.
7. Dewhirst MW, Tso CY, Oliver R, Gustafson CS, Secomb TW and Gross JF, Morphologic and hemodynamic comparison of tumor and healing normal tissue microvasculature. *Int J Radiat Oncol Biol Phys* **17:** 91–99, 1989.
8. Jain RK, Transport of molecules across tumor vasculature. *Cancer Metastasis Rev* **6:** 559–593, 1987.
9. Jain RK, Delivery of molecular and cellular medicine to solid tumors. *Adv Drug Delivery Rev* **26:** 71–90, 1997.
10. Folkman J, Addressing tumor blood vessels. *Nature Biotech* **15:** 510, 1997.
11. Folkman J, Anti-angiogenesis: New concept for therapy of solid tumors. *Ann Surg* **175:** 409–416, 1972.
12. Denekamp J, Vascular endothelium as the vulnerable element in tumours. *Acta Radiol Oncol* **23:** 217–225, 1984.
13. Huang X, Molema G, King S, Watkins L, Edgington TS and Thorpe PE, Tumor infarction in mice by antibody-directed targeting of tissue factor to tumor vasculature. *Science* **275:** 547–550, 1997.
14. Burrows FJ, Watanabe Y and Thorpe PE, A murine model for antibody-directed targeting to vascular endothelial cells in solid tumors. *Cancer Res* **52:** 5954–5962, 1992.
15. Burrows FJ and Thorpe PE, Eradication of large solid tumors in mice with an immunotoxin directed against tumor vasculature. *Proc Natl Acad Sci USA* **90:** 8996–9000, 1993.
16. Camerer E, Kolsto AB and Prydz H, Cell biology of tissue factor, the principal initiator of blood coagulation. *Thromb Res* **81:** 1–41, 1996.
17. Stone MJ, Ruf W, Miles DJ, Edgington TS and Wright PE, Recombinant soluble human tissue factor secreted by *Saccharomyces cerevisiae* and refolded from *Escherichia coli* inclusion bodies: Glycosylation of mutants, activity and physical characterization. *Biochem J* **310:** 605–614, 1995.
18. Hagemeier HH, Vollmer E, Goerdt S, Schulze-Osthoff K and Sorg C, A monoclonal antibody reacting with endothelial cells of budding vessels in tumors and inflammatory tissues, and non-reactive with normal adult tissues. *Int J Cancer* **38:** 481–488, 1986.
19. Schlingemann RO, Rietveld FJ, de Waal RM, Bradley NJ, Skene AI, Davies AJ, Greaves MF, Denekamp J and Ruiter DJ, Leukocyte antigen CD34 is expressed by a subset of cultured endothelial cells and on endothelial abluminal microprocesses in the tumor stroma. *Lab Invest* **62:** 690–696, 1990.
20. Rettig WJ, Garin-Chesa P, Healey JH, Su SL, Jaffe EA and Old LJ, Identification of endosialin, a cell surface glycoprotein of vascular endothelial cells in human cancer. *Proc Natl Acad Sci USA* **89:** 10832–10836, 1992.

21. Burrows FJ, Tazzari PL, Amlot P, Gazdar AF, Derbyshire EJ, King SW, Vitetta ES and Thorpe PE, Endoglin is an endothelial cell proliferation marker that is selectively expressed in tumor vasculature. *Clin Cancer Res* **1:** 1623–1634, 1995.
22. Griffioen AW, Coenen MJH, Damen CA, Hellwig SMM, van Weering DHJ, Vooys W, Blijham GH and Groenewegen G, CD44 is involved in tumor angiogenesis: An activation antigen on human endothelial cells. *Blood* **90:** 1150–1159, 1997.
23. Garin-Chesa P, Old LJ and Rettig WJ, Cell surface glycoprotein of reactive stromal fibroblasts as a potential antibody target in human epithelial cancers. *Proc Natl Acad Sci USA* **87:** 7235–7239, 1990.
24. Epstein AL, Khawli LA, Hornick JL and Taylor CR, Identification of a monoclonal antibody, TV-1, directed against the basement membrane of tumor vessels, and its use to enhance the delivery of macromolecules to tumors after conjugation with interleukin 2. *Cancer Res* **55:** 2673–2680, 1995.
25. Kairemo K, Ljunggren K, Strand SE, Hiltunen J, Penttila P, Nikula T, Laine A and Wahlstrom T, Radioimmunotherapy with 90Y-labeled monoclonal antibodies in a nude mouse ovarian cancer model. *Acta Oncol* **32:** 801–805, 1993.
26. Goldberg ID and Rosen EM (Eds.) *Regulation of Angiogenesis*, EXS Series, Vol. **79**. Birkhauser, Basel, 1997.
27. Folkman J, Watson K, Ingber D and Hanahan D, Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature* **339:** 58–61, 1989.
28. Battegay EJ, Angiogenesis: Mechanistic insights, neovascular diseases, and therapeutic prospects. *J Mol Med* **73:** 333–346, 1995.
29. Brooks PC, Role of integrins in angiogenesis. *Eur J Cancer* **32A:** 2423–2429, 1996.
30. Sato TN, Tozawa Y, Deutsch U, Wolburg-Buchholz K, Fujiwara Y, Gendron-Maguire M, Gridley T, Wolburg H, Risau W and Qin Y, Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* **376:** 70–74, 1995.
31. Stetler-Stevenson WG, Dynamics of matrix turnover during pathologic remodeling of the extracellular matrix. *Am J Pathol* **148:** 1345–1350, 1996.
32. Brooks PC, Clark RAF and Cheresh DA, Requirement of vascular integrin $\alpha_v\beta_3$ for angiogenesis. *Science* **264:** 569–571, 1994.
33. Yuan F, Chen Y, Dellian M, Safabakhsh N, Ferrara N and Jain RK, Time-dependent vascular regression and permeability changes in established human tumor xenografts induced by an anti-vascular endothelial growth factor/vascular permeability factor antibody. *Proc Natl Acad Sci USA* **93:** 14765–14770, 1996.
34. Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS and Ferrara N, Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. *Nature* **362:** 841–844, 1993.
35. Jakeman LB, Winer J, Bennett GL, Altar CA and Ferrara N, Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rat tissues. *J Clin Invest* **89:** 244–253, 1992.
36. Ferrara N and Keyt B, Vascular endothelial growth factor: Basic biology and clinical implications. *EXS* **79:** 209–232, 1997.
37. Brown LF, Detmar M, Claffey K, Nagy JA, Feng D, Dvorak AM and Dvorak HF, Vascular permeability factor/vascular endothelial growth factor: A multifunctional angiogenic cytokine. *EXS* **79:** 233–269, 1997.
38. Conforti G, Dominguez-Jimenez C, Zanetti A, Gimbrone MA, Cremona O, Marchisio PC and Dejana E, Human endothelial cells express integrin receptors on the luminal aspect of their membrane. *Blood* **80:** 437–446, 1992.
39. Brooks PC, Montgomery AMP, Rosenfeld M, Reisfeld RA, Hu T, Klier G and Cheresh DA, Integrin $\alpha_v\beta_3$ antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell* **79:** 1157–1164, 1994.
40. Brooks PC, Strömblad S, Klemke R, Visscher D, Sarkar FH and Cheresh DA, Antiintegrin αvβ3 blocks human breast cancer growth and angiogenesis in human skin. *J Clin Invest* **96:** 1815–1822, 1995.
41. Pasqualini R, Koivunen E and Ruoslahti E, αv Integrins as receptors for tumor targeting by circulating ligands. *Nature Biotech* **15:** 542–546, 1997.
42. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH and Folkman J, Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* **79:** 315–328, 1994.
43. Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonpierre PC and Yancopoulos GD, Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell* **87:** 1161–1169, 1996.
44. Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN and Yancopoulos GD, Angiopoietin-2, a natural antagonist for Tie2 that disrupts *in vivo* angiogenesis. *Science* **277:** 55–60, 1997.
45. Kroesen BJ, Helfrich W, Bakker A, Wubbena AS, Bakker H, Kal HB, The TH and de Leij L, Reduction of EGP-2-positive pulmonary metastases by bispecific-antibody-redirected T cells in an immunocompetent rat model. *Int J Cancer* **61:** 812–818, 1995.
46. Kroesen BJ, ter Haar A, Spakman H, Willemse P, Sleijfer DT, de Vries EGE, Mulder NH, Berendsen HH, Limburg PC, The TH and de Leij L, Local antitumour treatment in carcinoma patients with bispecific-monoclonal-antibody-redirected T cells. *Cancer Immunol Immunother* **37:** 400–407, 1993.
47. Schultz-Hector S and Haghayegh S, β-Fibroblast growth factor expression in human and murine squamous cell carcinomas and its relationship to regional endothelial cell proliferation. *Cancer Res* **53:** 1444–1449, 1993.
48. Leek RD, Harris AL and Lewis CE, Cytokine networks in solid human tumors: Regulation of angiogenesis. *J Leukoc Biol* **56:** 423–435, 1994.
49. O'Reilly MS, Holmgren L, Chen C and Folkman J, Angiostatin induces and sustains dormancy of human primary tumors in mice. *Nature Med* **2:** 689–692, 1996.
50. Viola A and Lanzavecchia A, T cell activation determined by T cell receptor number and tunable thresholds. *Science* **273:** 104–106, 1996.
51. Thieme H, Aiello LP, Takagi H, Ferrara N and King GL, Comparative analysis of vascular endothelial growth factor receptors on retinal and aortic vascular endothelial cells. *Diabetes* **44:** 98–103, 1995.
52. Boucher Y, Salehi H, Witwer B, Harsh GR 4th and Jain RK, Interstitial fluid pressure in intracranial tumours in patients and in rodents. *Br J Cancer* **75:** 829–836, 1997.
53. Pratesi G, Rodolfo M, Rovetta G and Parmiani G, Role of T cells and tumour necrosis factor in antitumour activity and toxicity of flavone acetic acid. *Eur J Cancer* **26:** 1079–1083, 1990.
54. Harris SR and Thorgeirsson UP, Flavone acetic acid stimulates nitric oxide and peroxynitrite production in subcutaneous mouse tumors. *Biochem Biophys Res Commun* **235:** 509–514, 1997.
55. O'Reilly SM, Rustin GJ, Farmer K, Burke M, Hill S and Denekamp J, Flavone acetic acid (FAA) with recombinant interleukin-2 (rIL-2) in advanced malignant melanoma: I. Clinical and vascular studies. *Br J Cancer* **67:** 1342–1345, 1993.