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Review Article

Tumor vascular targeting

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Abstract

Conventional tumor therapy typically suffers from a lack of specificity, leading to a poor therapeutic index and adverse events. The selective delivery of bioactive molecules to tumor sites represents an attractive therapeutic strategy, which promises to spare normal organs and yield medicinal agents with improved therapeutic ratios. For targeting the tumor environment, newly formed blood vessels represent an ideal target for the delivery of bioactive molecules, since they are readily accessible from the bloodstream and are different at a molecular and morphological level from the normal vasculature. Tumors can not grow beyond a certain size without establishing a blood supply of their own. As such, angiogenesis, or the formation of new blood vessels, represents a therapeutically interesting concept, as in adults this process is essentially limited to the tumor environment. Considerable effort has already been directed towards the development of agents (so-called antiangiogenics) capable of interfering with the process of neovascularization. It has now become evident that the targeted delivery of bioactive molecules to the tumor neovasculature represents a distinct, attractive avenue for biomedical intervention. This review presents evidence that tumor vascular targeting is an effective therapeutic strategy in animal models, describes the means of discovering new vascular targets and discusses the current and future of tumor vascular targeting activities in the clinical setting.

Introduction

The chemotherapy of cancer often relies on the assumption that drugs will preferentially kill rapidly growing tumor cells rather than cells in normal tissues. However, most conventional anticancer drugs display a lack of specificity, leading to an overall low therapeutic index and toxicity to healthy organs with enhanced proliferation rates, such as hair follicles, bone marrow and the gastrointestinal tract.

Chemotherapeutic drugs typically exhibit low accumulation in the tumor, mainly due to the markedly irregular, tortuous vasculature and high interstitial blood pressure that are inherent characteristics of the tumor environment (1). Moreover, multidrug resistance (MDR) proteins may further decrease drug uptake and can even cause therapy failure (2). Consequently, the development of agents that are capable of selectively accumulating in solid tumors represents an exciting new field and is a key goal of modern anticancer research.

Tumor vascular targeting comprises the targeted delivery of bioactive molecules, such as drugs, cytokines, radionuclides or procoagulant factors, to the tumor environment through the use of binding molecules specific for tumor-associated markers. Figure 1 illustrates this general concept.

The endothelium and surrounding stroma of tumors are quite different from normal tissues at a molecular, anatomical and pathophysiological level. For example, the tumor vasculature is remarkably disorganized and tortuous. Indeed, it is sometimes difficult to distinguish arterioles and venules, and the occurrence of vascular shunts, in which blood passes directly from an arteriole to a venule, is common. Moreover, the flow of blood through the tumor capillaries is often sluggish, and at times may be stationary or even experience a reversal in the direction of flow (3, 4). The hemoglobin in the erythrocytes is depleted of oxygen and the microenvironment, which includes both the blood and the endothelium lining the vessels, is profoundly hypoxic (5).

In this environment, the endothelial cell is distinctly different from that in normal tissue, in which the endothelium is remarkably quiescent. The endothelium in tumors proliferates rapidly and contributes to active angiogenesis

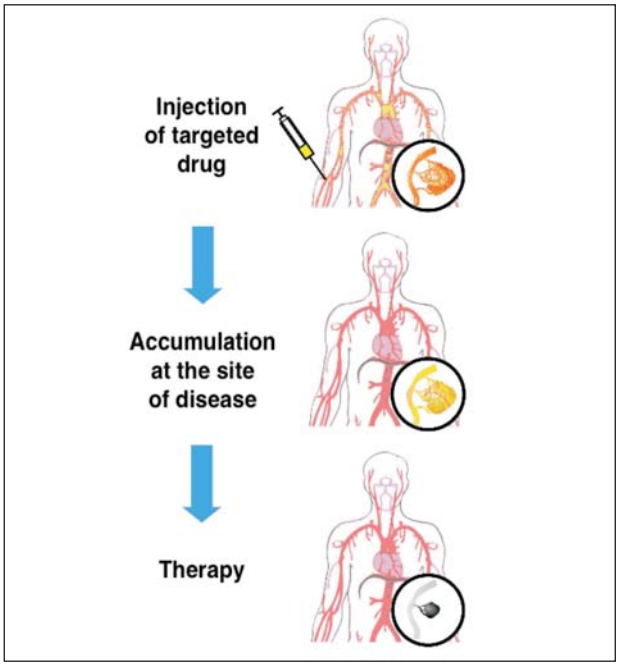

Fig. 1. Concept of ligand-based vascular targeting of disease. The targeted drug, consisting of a ligand and an effector function, is administered intravenously. The ligand binds specifically to a marker, which is overexpressed and accessible from the bloodstream in the diseased tissue (*e.g.,* a tumor), but not (or only to a much lesser extend) present or accessible in normal, healthy organs. This leads to an accumulation of the drug at the site of disease. If the effector function is an imaging agent, this accumulation allows the detection of the lesion(s) by diagnostic procedures. In the case of a therapeutic effector function, the drug will preferentially act at the site of disease, which reduces side effects to healthy tissues.

(6). The tumor is also nutrient (*e.g.*, glucose)-starved, acidic (owing to lactate production from anaerobic glycolysis) and under oxidative stress (7, 8). The endothelial cell has been shown to respond transcriptionally to all these stimuli, and the existence of hypoxically induced endothelium-specific genes indicates that the endothelial cell can adapt to a hypoxic environment.

While the term “vascular tumor targeting” is often used to denominate a broad variety of anticancer strategies that affect the endothelium (*e.g.,* the use of combretastatins to achieve intraluminal blood coagulation at the tumor site), in this review we will only focus on agents capable of selective localization in the tumor neovasculature. Such molecules represent invaluable vehicles for the efficient delivery of bioactive molecules to the tumor environment.

Identification of tumor vascular targets

Until recently, most approaches for the identification of tumor vascular targets relied on the direct comparison of endothelial cells cultured *in vitro* under conditions thought to mimic those in normal and tumor tissues. The culture conditions included those favoring proliferation or quiescence, hypoxia or normoxia, as well as exposure to tumor cell-conditioned media. Protein electrophoresis clearly revealed differences in gene expression (9), yet it proved difficult to identify the differentially expressed proteins at the molecular level. Another popular approach has been to raise antibodies, either by immunization (10) or by antibody phage technology (11), to different endothelial cell cultures. These efforts were successful in terms of the discovery of endothelial markers, but failed to identify differentially expressed genes, possibly because such proteins represent a minority of all proteins present on the cell surface. However, real advances have come with the emergence of techniques that allow full genome analysis. In particular, mRNA-based serial analysis of gene expression (SAGE) or microarray analysis combined with bioinformatics analyses on the large quantity of expression data that are now available in the public domain have been especially fruitful.

Bioinformatics

In 1995, Adams and colleagues presented an initial assessment of human gene diversity and expression patterns based on 83 million nucleotides of DNA sequences (12). They observed that the endothelial cell is one of the richest transcriptional sites, which indicates that there could be several genes for which expression is restricted to the endothelium. Their work comprised the selection of tumor endothelial markers by screening the expression of endothelium-specific genes in normal and tumor tissues. One such approach applied a subtractive algorithm to the expressed sequence tag expression data available in public databases to identify novel endothelium-specific genes (13). These were then screened for expression by *in situ* hybridization, which identified roundabout-4 (ROBO4) and an endothelium-specific protein, disulfide isomerase (EndoPDI), as tumor endothelial markers (14, 15).

Transcriptomics

Serial analysis of gene expression, or SAGE, allows simultaneous and quantitative analysis of a large number of transcripts (16). Following the isolation of endothelium from normal and cancerous colon, SAGE libraries were constructed and a direct comparison identified genes that were upregulated in the tumor endothelium, thus leading to the identification of several novel tumor endothelial markers, or TEMs (17). As target accessibility from the bloodstream is fundamental in tumor vascular targeting, the work was pursued on proteins with predicted transmembrane domains (17-19).

TEM1, TEM7 and TEM8 all show a single-pass transmembrane domain, whereas TEM5 is an orphan seven-pass transmembrane G-protein-coupled receptor (GPCR) with a long extracellular amino-terminal domain that belongs to the adhesion family of GPCRs. An investigation performed on the expression of mouse orthologues in normal and tumor tissue revealed the exclusive expres-

sion of TEM1, TEM5 and TEM8 in tumor endothelium (18). TEM1 has been shown to be identical to endosialin (20), suggesting a contamination of the endothelial cells from which the SAGE libraries were derived with pericytes and fibroblasts, which represent the main source of this marker in normal conditions (21). TEM8 has been shown to be an anthrax toxin receptor, which might explain the antitumor activity of the toxin, as binding of the toxin to TEM8 expressed on tumor endothelium leads to endothelial death (22).

Perfusion with silica beads

The *in vivo* labeling of vasculature, followed by recovery and comparative proteomic analysis, constitutes a very promising avenue for the discovery of novel markers of angiogenesis. Schnitzer *et al.* used a subtractive proteomic mapping strategy to identify proteins that are differentially expressed on the endothelial surface in normal and tumor tissue (23). They described the use of colloidal silica for the *in vivo* coating of vascular structures, followed by subcellular fractionation to directly isolate luminal endothelial cell plasma membranes. The isolated plasma membranes are then analyzed using two-dimensional gel electrophoresis or multiple multidimensional mass spectrometry techniques to produce high-resolution protein maps. Differential spot analysis followed by mass spectrometry of tryptic peptides, database searching and immunoblotting allowed the characterization of differentially expressed proteins. Indeed, the analysis of the endothelium of normal rat lung tissue compared to lungs with metastatic breast adenocarcinoma led to the identification of 15 proteins that were upregulated on the endothelium (24). One of these markers, annexin A1, will be described below.

In vivo and ex vivo biotinylation

Recently, our group developed a methodology based on the *in vivo* perfusion of tumor-bearing mice with active ester derivatives of biotin (25). The active ester moiety of the biotinylation reagent subsequently reacts with primary amino groups of proteins, which are readily accessible from the bloodstream. After homogenization in the presence of SDS, the biotinylated proteins were captured onto a streptavidin column, subjected to tryptic digestion and identified using LC-MS/MS methodologies (26, 27). The biotinylation of mice with F9 subcutaneous tumors or orthotopic kidney tumors revealed both quantitative and qualitative differences in the recovery of biotinylated proteins compared to normal tissues (25). This approach has lately been extended to the *ex vivo* biotinylation of surgically resected human organs with cancer (28).

Targets of tumor vasculature

Several protein antigens expressed either in the tumor blood vessel or in the adjacent matrix have been characterized through *in vivo* targeting studies using radiolabeled targeting agents, or molecules delivering a payload to the tumor vasculature. In principle, marker proteins expressed on the luminal surface of endothelial cells promise to be the most readily accessible targets for intravenously administered ligands. However, antigens expressed in the stroma around the neovasculature have the advantage of being typically more abundant and more stable in a variety of different tumor types.

Extra domain B (ED-B) of fibronectin

Fibronectin is a large glycoprotein present in considerable amounts in plasma and tissues. ED-B is a 91-amino-acid type III homology domain that becomes inserted into the fibronectin molecule during tissue remodeling by a mechanism of alternative splicing at the level of the primary transcript (29). In healthy adult individuals, ED-B is essentially undetectable. However, ED-B-containing fibronectin is abundant in many aggressive solid tumors and displays either predominant vascular or stromal expression patterns, depending on the tumor type (30).

As the ED-B sequence is identical in mice, rats, rabbits, dogs, monkeys and man, the generation of anti-ED-B antibodies using hybridoma technology has not been possible, probably due to tolerance. However, the conservation of the ED-B domain facilitates animal experiments in immunocompetent syngeneic settings using human monoclonal phage-derived antibodies (31-33). These include the high-affinity human antibody L19, which was isolated in our laboratory and has been shown to efficiently localize on tumor blood vessels in animal models (34-37) and patients with cancer following intravenous injection (38). The three-dimensional structure of ED-B has been solved in solution using nuclear magnetic resonance (NMR) methods (39). A large number of therapeutic derivatives of the L19 antibody have been produced. Notably, the anti-ED-B antibody L19 in homodimeric scFv format and labeled with iodine-123 has been studied in over 40 patients with cancer. The results obtained in the first 20 patients were recently described (38) and confirmed the ability of the antibody to localize to tumor masses exhibiting rapid growth. An immunohistochemical analysis using L19 in small immunoprotein (SIP) format showed strong staining of the human xenograft tumor A375 (malignant melanoma) (Fig. 2).

Large tenascin-C isoforms

Tenascins are a family of four glycoproteins that are typically present in many different connective tissues, where they contribute to extracellular matrix (ECM) structure and influence the behavior of cells that are in contact with the latter. Tenascins are all built from a common set of structural motifs, and several isoforms of the protein can be generated as a result of different patterns of alternative splicing in the region between fibronectin type III domains A1 and D.

Certain large extra domain-containing isoforms have been identified as tumor-associated antigens, showing if

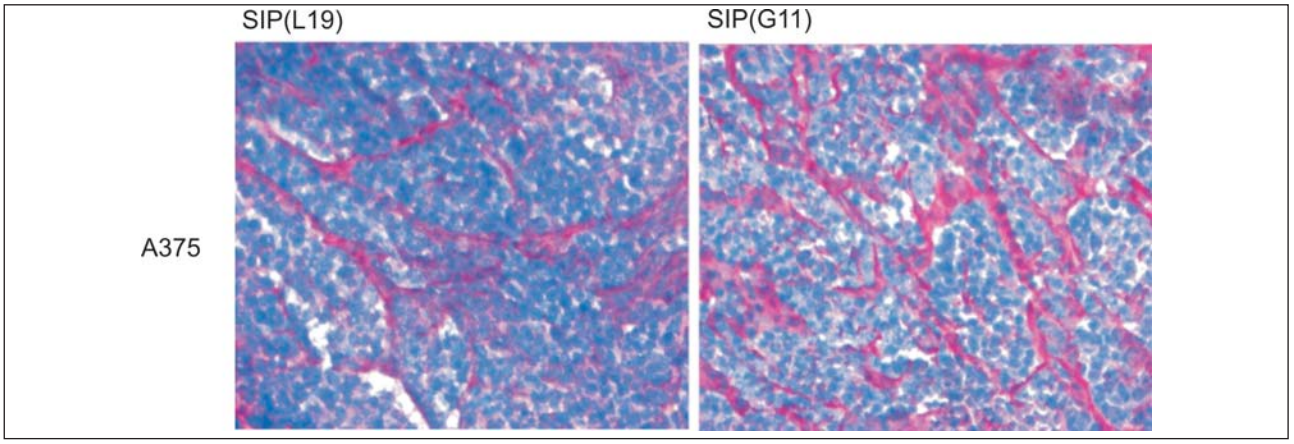

Fig. 2. Immunohistochemical analysis of human A375 malignant melanoma xenograft. The sections were stained with SIP(L19) (left panel) and SIP(G11) (right panel).

not a complete absence, a clearly more restricted expression pattern in normal tissues compared to the small tenascin isoforms without extra domains (40). Of all domains that can be inserted in the large isoforms, domain C of tenascin-C shows the most restricted expression pattern. While being undetectable in normal human tissues and only barely detectable in most carcinomas, it is extremely abundant in high-grade astrocytoma (grade III and glioblastoma), particularly around vascular structures and proliferating cells (41). Immunohistochemical experiments using G11, a phage-derived antibody targeting the extra domain C of tenascin-C, in SIP format showed a prominent perivascular staining pattern in A375 xenografts (Fig. 2).

A critical immunohistochemical analysis of the expression pattern of the different isoforms in various cancer types is required to evaluate their potential as targets for biomolecular intervention. Radiolabeled derivatives of monoclonal antibodies to domains A1 and D of tenascin-C have been used for over a decade for imaging and radioimmunotherapy in patients with cancer (42, 43). The staining pattern of these antibodies varies between different tumors, the two extremes being a predominantly vascular and a diffuse stromal staining.

Vascular endothelial growth factors (VEGFs) and their receptors

VEGFs represent a class of proteins that mediate angiogenesis, increase vascular permeability and contribute to endothelial cell survival in blood and lymphatic vessels (44-47). The overexpression of VEGFs and their receptors in tumors (24, 48) makes them attractive antigens for the targeted delivery of antibody derivatives to the tumor neovasculature (49). The contribution of VEGF to cancer progression is further highlighted by the recent approval of the humanized anti-VEGF monoclonal antibody bevacizumab (Avastin®; Genentech) for first-line cancer treatment (44, 50).

In addition, the selective localization of monoclonal antibodies to VEGFR-2 and the VEGF-A/VEGFR-2 complex has been studied (51). The targeting efficiencies reported so far were modest, which possibly reflects kinetic limitations in the targeting of low- or medium-abundance antigens, even when they are readily accessible to intravenously injected binding agents (52).

Integrins

During vascular remodeling and angiogenesis, endothelial cells exhibit increased expression of several cell-surface molecules that potentiate cell invasion and proliferation. One such molecule is integrin $\alpha_v\beta_3$, which has a key role in endothelial cell survival during angiogenesis *in vivo* and which might be suitable as a target for therapeutic molecules, particularly those that require internalization in endothelial cells. $\alpha_v\beta_3$ was the first integrin shown to be associated with tumor angiogenesis and is indeed highly expressed in angiogenic endothelial cells in malignant tumors and wound granulation tissue, but not, or to a much lower extent, in quiescent endothelial cells.

A high-affinity anti-$\alpha_v\beta_3$ humanized antibody (LM609, Vitaxin®; MedImmune) is in clinical development as an antiangiogenic therapeutic agent (53). However, so far its tumor-targeting performance for cancer imaging has been unsatisfactory (54). Anti-$\alpha_v\beta_3$ antibodies have been shown to preferentially localize on tumor blood vessels using *ex vivo* fluorescence microscopy detection (55). Moreover, a paramagnetic contrast agent targeted to the LM609 monoclonal antibody, which is specific for $\alpha_v\beta_3$, has been described for the *in vivo* imaging of angiogenesis using magnetic resonance (56).

Annexin A1

Annexins are cytosolic proteins capable of associating with the cell membrane in a calcium-dependent manner. Some annexins might translocate the lipid bilayer to the external cell surface. As described earlier, Schnitzer *et al.* recently discovered annexin A1 as a tumor endothelial target by the terminal perfusion of tumor-bearing rats with silica beads. A monoclonal antibody to annexin A1 has

been used for the radioimmunoscintigraphic detection of solid tumor lesions in a rat model. Moreover, relatively low radioactive doses of the same antibody labeled with iodine-125 showed therapeutic benefit in rats (24).

Prostate-specific membrane antigen (PSMA)

PSMA is a membrane glycoprotein with proteolytic activity. It is predominantly expressed in the prostate and serum concentrations are often elevated in patients with prostate cancer (57). There have been several studies showing an overexpression of PSMA in the neovasculature of various tumors (58, 59), whereas in normal vasculature the expression is restricted to some vessels of the breast, duodenum, kidney and prostate. Tumor imaging using the radiolabeled humanized monoclonal antibody J591 demonstrated excellent tumor targeting (60), and the antibody is currently being evaluated for therapeutic applications (61, 62). Furthermore, radioimmunotherapy studies in rodent models of cancer using radiolabeled derivatives of J591 with α-emitters have shown antiproliferative activity (63). Due to their high energy and short tissue penetration (a few cell layers), α-particles might be uniquely suited as selective depositors of toxic radiation to tumor vascular structures (64).

Specific binding molecules for vascular targeting applications

At present, monoclonal antibodies are the best-established class of binding molecules for tumor diagnosis and therapy. Nonetheless, certain drawbacks do exist, such as the requirement for an expensive mammalian cell production system, dependence on disulfide bonds for stability, low expression yields and a tendency for aggregation, especially when fused to additional domains or proteins (*e.g.,* cytokines). Therefore, aptamers, peptides and specific small organic molecules are currently being investigated for tumor targeting applications.

Antibodies

Monoclonal antibodies (and possibly aptamers) represent the only classes of specific binding molecules that can be rapidly isolated and that exhibit strong affinity for virtually any antigen. Already in 1975, Kohler and Milstein succeeded in isolating monoclonal antibodies of rodent origin by means of hybridoma technology (65). In 1986, the group of Greg Winter pioneered the generation of humanized antibodies by grafting the complementarity-determining regions (CDRs) of murine antibodies onto a human antibody framework (66), thereby minimizing immunogenicity. The antibody humanization methodology was later complemented by the generation of human antibodies through immunization of transgenic animals carrying human immunoglobulin genes (67) and by antibody phage technology (68).

The construction of large ($> 10^9$) libraries of human antibodies is possible through the display of antibody fragments (scFv or Fab) on the surface of a filamentous phage (69, 70). Monoclonal antibodies can then be isolated by panning the phage library onto the antigen of choice, and selected antibody fragments may be affinity-matured using combinatorial mutagenesis of the antibody gene in order to achieve dissociation constants in the nanomolar to picomolar range (31). In 1999, ribosome display was proposed as a fully *in vitro* avenue for the isolation and affinity maturation of human antibodies (71).

The available array of antibody formats shows different pharmacokinetics and tumor targeting properties (34). Full IgGs are slowly eliminated from the blood and typically accumulate in the liver. Consequently, rapidly clearing antibody fragments are typically preferred for imaging applications in nuclear medicine. However, intact immunoglobulins remain the antibody format of choice for many therapeutic applications (72), such as whenever activation of signaling pathways, the complement system or antibody-dependent cellular cytotoxicity (ADCC) is required for therapeutic benefit. If desired, antibody fragments isolated by phage display may easily be converted to full IgGs by subcloning the genes encoding the variable antibody domains into a suitable expression vector.

Aptamers

In addition to antibodies, aptamers (single-stranded nucleic acids capable of adopting a complex three-dimensional structure) are possibly the only other class of molecules from which specific binding molecules against a variety of target proteins can be rapidly isolated (73). Aptamer technology enables the generation of large libraries ($> 10^{12}$) of single-stranded DNA or RNA molecules, which can be panned for target antigen binding. The nucleic acids selected during this procedure are then amplified using PCR-based techniques in order to generate the input material for further selection cycles. Aptamers are typically molecules of 8-15 kDa, making them slightly smaller than scFvs (25 kDa) and intermediate in size compared to antibodies (150 kDa) and small peptides (1-5 kDa). The rather small size and polyanionic nature of aptamers may minimize the residence in excretory organs, providing potentially useful features for imaging and radiotherapy, but it is unclear whether they may also impact the ability of the aptamer to extravasate. Dissociation constants of aptamers usually lie in the micromolar to subnanomolar range. The *in vivo* stability still needs to be properly assessed and a number of strategies have been proposed for this purpose, including Spiegelmer technology (74).

Recently, the first therapeutic aptamer, pegaptanib (Macugen®; Pfizer, Eyetech), was approved for the treatment of age-related macular degeneration (AMD) (75). Upon intravitreal administration, pegaptanib binds to VEGF-165 (but not to smaller VEGF-A isoforms) and inhibits ocular angiogenesis. Promising imaging studies in rodent models of cancer with the radiolabeled aptamer TTA1, specific for tenascin-C, have recently been described (76). The potential of aptamers for tumor tar-

geting applications is currently being investigated in clinical trials.

Peptides

Peptidic binders to target proteins of interest can be isolated from commercially available phage display libraries of linear and disulfide constraint peptides (77). However, peptide phage display generally has lower success rates than antibody phage technology and yields binders of affinities that rarely exceed the micromolar range. This limitation can be overcome by multimerization, resulting in an improvement of avidity (78, 79). Ribosome display (80) and other technologies for the construction of very large peptide libraries and their molecular evolution have been proposed (81), but the isolation of high-affinity peptidic binders remains a difficult challenge. Peptide phage libraries have been used for *in vivo* panning by the groups of Ruoslahti and Pasqualini (82, 83), but there is still a need to confirm the real imaging and therapeutic potential of these phage-derived peptides in advanced animal models and the clinic.

Several internalizing peptides with specificity for receptors that are overexpressed on the tumor cell have been used for the imaging of tumors and the selective delivery of therapeutic radionuclides to neoplastic lesions. For example, the somatostatin analogue octreotide (Sandostatin® LAR®; Novartis) has been approved in Europe and the U.S. for the imaging of tumors (84). A number of other agents are in development (85, 86), such as integrin-binding peptides comprising an arginine-glycine-aspartate (RGD) tripeptide motif (87) or bombesin peptide analogues (87, 88). Moreover, naturally occurring peptides, *e.g*., peptides derived from physiological angiogenesis-inhibitory proteins or resulting from the degradation of extracellular matrix components, have been shown to selectively target the tumor vasculature using microscopic techniques (89). The introduction of an analogy to the CDR loops of binding antibodies has also been exploited for peptide design with the aim of constructing tumor-targeting molecules with improved specificity and affinity (90). Nevertheless, the generation of peptidic binders of sufficient affinity is not possible for every antigen. Additionally, the limited *in vivo* stability of peptidic drugs remains a major cause of concern.

Small organic molecules

The majority of drugs on the market are small organic compounds. Such molecules have several advantages over biopharmaceuticals, including oral bioavailability, ease of manufacture, lack of immunogenicity and favorable tissue distribution properties. While typical dissociation constants can be nanomolar for antigens with cavities (*e.g*., enzymes), the isolation of high-affinity small organic binders to flat protein surfaces remains a major challenge (91). Experimental evidence exists suggesting that affinity and specificity can be increased by exploiting the chelate effect by means of linking two or more organic compounds that recognize adjacent epitopes on the target antigen (92). Methods for the identification of such bidentate ligands include “SAR by NMR” (93), dynamic combinatorial chemistry (94) and tethering approaches (95).

Recently, this list was expanded by an approach termed Encoded Self-Assembling Chemical (ESAC) libraries developed in our laboratory (96, 97). This technology allows the facile construction of very large libraries of chemical compounds by the DNA-mediated self-assembly of smaller sublibraries. Each pharmacophore in the library is covalently coupled to an oligonucleotide, which mediates the self-assembly of the library and provides the pharmacophore with a distinctive DNA identification tag. As for antibody phage display libraries, ESAC libraries can be panned in solution, thus enriching bidentate ligands that display preferential binding to the target of interest. The decoding of the selected compounds can be performed by a number of experimental techniques, including an asymmetric PCR followed by sequencing or hybridization onto a DNA chip. We have described the isolation of ESAC-derived bidentate molecules with nanomolar affinity for carbonic anhydrase (96), which has been identified as an interesting disease target. Indeed, in renal cell carcinoma, carbonic anhydrase IX can be upregulated under hypoxic conditions (98).

At present, it appears that combinatorial chemistry methodologies such as Speed Screen (99) and DNA-encoded chemical library technology (100) may allow the screening of chemical libraries of unprecedented size. It remains to be seen whether these approaches are sufficiently efficient in generating specific binders to protein targets of interest to compete with recombinant antibody technology.

Use of ligands for selective delivery of bioactive moieties to tumor site

Imaging applications

The macroscopic *in vivo* imaging of sites of disease is perhaps one of the most straightforward biomedical applications of ligands (typically monoclonal antibodies) capable of selective localization around tumor vascular structures. The attractiveness of antibody-based vascular targeting for imaging applications lies in the ready accessibility of vascular structures for agents that are administered intravenously. However, the targeting of different classes of vascular antigens can be more or less efficient, depending on whether the antigens are expressed on the luminal or abluminal side of new blood vessels (101). Moreover, the same antigen can be expressed on either of these aspects of new blood vessels in different tumors (*e.g*., luminal expression of ED-B in glioblastoma) (102).

Luminal antigens can perhaps be considered superior for imaging applications, yet this may not always be the case. For example, luminal antigens are often less abundant than other antigens (*e.g.,* components of the basement membrane), necessitating the use of low concentrations of ligands in order to achieve a stoichiometric

match between target and targeting agent (52). Consequently, low concentrations of ligand will require targeting agents of exceptionally high affinity, which may nonetheless be incapable of selective targeting since association kinetics are limited by diffusion (*i.e.*, upper limit on kinetic association constants).

For the majority of practical molecular imaging applications, four main modalities can be considered: radioactivity-based imaging procedures (positron emission tomography [PET]/single photon emission computed tomography [SPECT]), near-infrared (NIR) fluorescence imaging, magnetic resonance imaging **(**MRI) and ultrasound-based imaging.

Most imaging applications of vascular targeting antibodies have been performed using radiolabeled antibody preparations. We have recently reviewed these experiments elsewhere (103). The increasing relevance of PET/CT imaging methodologies, with their excellent sensitivity, resolution and possibility of quantification, highlights the need for suitable labeling strategies for antibody fragments with PET radionuclides.

NIR fluorescence imaging is based on the fact that infrared radiation can penetrate tissues to a certain extent, with 10% penetration through 1 cm at 800 nm (104, 105). Tomographic procedures such as diffuse optical tomography have recently made it possible to detect breast cancer lesions 5-10 cm below the skin using nonspecific fluorophores as contrast agents (106). Furthermore, it is expected that the targeted delivery of judiciously selected fluorophores to sites of disease may facilitate the use of NIR imaging modalities in biomedical applications.

Due to its high resolution and no need for the use of radioactivity, MRI would be an ideal methodology for molecular imaging applications. However, MRI contrast agents usually need to be used at high concentrations. For instance, gadopentetate dimeglumine (Gd-DTPA, Magnevist®) is typically administered at 0.1 mmol/kg, enabling sufficient relaxivities for minutes to hours, depending on the elimination rate, at blood concentrations in the range of 240-600 μM. Estimations show that local concentrations of Gd in the order of 1-10 μM are needed to provide sufficient sensitivity for MRI-based imaging applications. Such conditions may be met only for the most abundant luminal vascular antigens and will likely require the use of immunoliposomes (56) or antibody-nanoparticle conjugates (107). It is uncertain whether these large molecules will be able to extravasate, thus accessing perivascular structures. Such concerns also exist for microbubble-based ultrasound contrast agents, even though the successful use of peptide microbubble conjugates for the imaging of tumor vasculature has recently been reported (108). The use of anti-ED-B antibody-microbubble conjugates has until now only been described *in vitro* (109).

Therapeutic applications

Presently, unmodified antibodies in IgG format represent the large majority of antibody-based products approved for clinical use in the U.S. and Europe. Nevertheless, two radiolabeled antibodies (Zevalin® from Biogen Idec and Bexxar® from GlaxoSmithKline) and one drug conjugate (Mylotarg®; Wyeth) have been approved for cancer therapy. It is very likely that in the future more antibody derivatives in which the antibody moiety acts as a vehicle to deliver therapeutic agents at sites of disease will be used clinically.

The modification of the structure of an intact antibody or antibody fragment can result in considerable changes in pharmacokinetic behavior. At the two extremes, full IgGs display long residence time in blood (34), while the small scFv fragments are rapidly cleared via the renal route, leading to clearance of > 90% of the injected antibody from blood within 1 h (36). Much effort is put into the pharmaceutical development of intact antibodies, and recent advances in the potentiation of Fc-mediated effector functions by mutagenesis (110) or glycoengineering (111) are likely to improve the therapeutic performance of antibodies that are already used in the clinic. In particular, a judicious engineering of the Fc portion of the antibody molecule can influence both complement activation and ADCC. Conversely, scFv antibody fragments may be preferable for the delivery of bioactive agents at sites of disease, since these proteins retain the binding specificity of the parental antibody (112) and are encoded by a single gene, thus facilitating functionalization strategies based on genetic fusion.

Figure 3 illustrates a selection of the most promising antibody modification strategies that are being pursued clinically or preclinically. These are typically grouped in two main categories, depending on whether the antibody derivative is to be used for imaging or therapeutic applications. For therapeutic applications, antibody fragments have successfully been coupled to fluorophores and photosensitizers (105, 113, 114), radionuclides (34, 103, 115), liposomes (116, 117), procoagulant agents (118, 119), cytokines (52, 120-123), enzymes (124) and other proteins (101, 125). Some of the most promising conjugation partners will be presented in the following section.

1. Cytokines

Proinflammatory cytokines such as interleukin-2 (IL-2) or tumor necrosis factor (TNF) have been used in the therapy of certain tumors for some time. However, their very small therapeutic window and high toxicity has led them to be used solely in tumors which can not be treated by other means (126), or in isolated limb perfusion (127). IL-12, another member of this group, never found its way into routine clinical application due to extremely high toxicity and even deaths in a phase II clinical trial (128). Antibody-cytokine fusion proteins allow the pursuit of a logical avenue for improving the therapeutic index of anticancer cytokines whose excessive toxicity compromises their clinical value (129). Fusion proteins composed of the scFv antibody fragment L19 and either IL-2 or TNF exhibited excellent tumor uptake and potent antitumor activity in various animal models (120, 122). Both

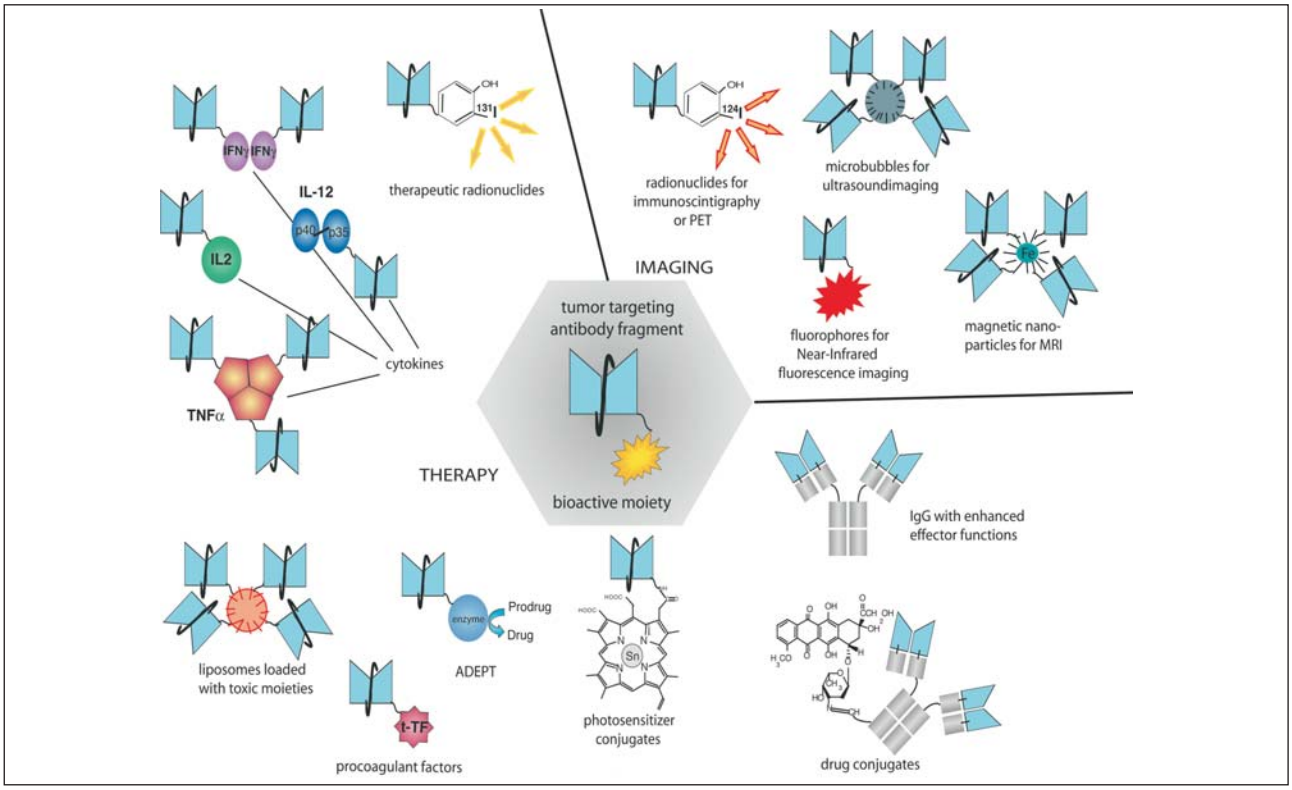

Fig. 3. Schematic representation of antibody derivatives that can be used either for tumor imaging or therapeutic applications.

L19-IL-2 and L19-TNF are currently in clinical development (130).

The fusion protein L19-IL-12 showed promising tumor therapy results, in spite of modest performance in biodistribution studies and especially when combined with L19-TNF (121). Recent protein-engineering experiments have permitted a considerable potentiation of the tumor-targeting and therapeutic activity of L19-IL-12 fusions. The fusion of a murine interferon gamma mutant with scFv(L19) resulted in a bioactive protein that accumulated at the tumor site and exhibited a strong antitumor effect, which could be further enhanced by combination with other immunocytokines and doxorubicin (123).

2. Tissue factor

The selective delivery of procoagulant factors to the tumor neovasculature is a promising approach for depriving tumor cells of oxygen and nutrients. Thorpe *et al.* examined the antitumor performance of truncated tissue factor (tTF) that was selectively delivered to the vascular endothelium in tumors through the use of antibodies to either vascular cell adhesion molecule-1 (VCAM-1) or to an artificially introduced antigen (131). The results were promising and showed retardation of tumor growth or even total collapse of the tumor. The fusion of tTF to L19 resulted in a fusion protein that accumulated around tumor blood vessels *in vivo* and even caused the complete and selective infarction of three different types of solid tumors in mice (118).

3. Photosensitizers

Photodynamic therapy (PDT) is a promising method for the treatment of superficial tumors. However, the lack of selectivity of photosensitizers results in severe toxicity, which limits the usefulness of the approach. Such agents were conjugated to antibodies for targeted delivery to tumor sites and subsequently activated *in situ* with light of the corresponding wavelength, which resulted in encouraging antitumor responses in animal models (132). As for TF, this strategy also aims at the selective occlusion of blood vessels, thereby depriving tumors of nutrients and oxygen, and ultimately causing tumor cell death. The chemical conjugation of the photosensitizer bis(triethanolamine)Sn(IV)chlorine6 to L19 and subsequent irradiation with red light produced complete and selective occlusion of ocular neovasculature and promoted apoptosis of the corresponding endothelial cells (113). Mice bearing different subcutaneous tumors were subjected to treatment with the same photosensitizer coupled to SIP(L19), which resulted in considerable tumor growth retardation (133).

4. Radioligands

Recently, the approval of Zevalin® and Bexxar®, two radiolabeled anti-CD20 antibodies for the radioimmunotherapy (RIT) of lymphoma, spurred a renewed interest in radioimmunotherapy as an avenue for the selective eradication of disseminated neoplasia (134).

Some of the attractions of RIT strategies are the cross-fire effect of β-emitting radionuclides and the possibility to predict therapeutic outcome based on dosimetric data. However, the practical implementation of RIT protocols in the clinic remains problematic due to cost issues and the need for radioprotection. Furthermore, there are concerns about long-term side effects of antibody-based radiopharmaceuticals, particularly if they are cleared via the renal route (135).

Similar approaches could be considered for vascular targeting strategies (136). Van Dongen *et al.* described antitumor effects in incurable patients with bulky head and neck squamous cell carcinoma treated with a ^{186}Re-labeled humanized monoclonal antibody to CD44v6 (137). As shown for the anti-PSMA antibody J591, α-emitters are also ideally suited for being coupled to vascular targeting agents, as their high energy and short tissue penetration would selectively destroy tumor endothelial cells (63). The L19 antibody derivative ^{131}I-[SIP]-L19 exhibited a superior antitumor efficacy and toxicity profile in the F9 teratocarcinoma mouse model compared to other L19-based radioimmunoconjugates (115). Clinical trials investigating ^{131}I-[SIP]-L19 in patients with cancer are in progress.

5. Drugs

Antibody-drug conjugates represent another important class of therapeutic agents. One such agent, Mylotarg®, comprised of an anti-CD33 antibody and the cytotoxic drug calicheamycin, has been approved for the treatment of acute myeloid leukemia (AML). Currently, most efforts in this field are being made by biotech companies such as Immunogen, Seattle Genetics and Genentech (138-140).

Summary and outlook

Strategies based on ligands capable of targeting the tumor vasculature offer a unique opportunity for delivering high concentrations of therapeutic agents at sites of disease. In preclinical models, this has led to impressive therapeutic effects, especially in the field of tumor-targeting immunocytokines and radiolabeled antibodies. The first products of these classes are currently being investigated in clinical trials.

Antibodies and immunoconjugates are advancing in their role as highly selective and efficient therapeutics for the treatment of cancer. Compared with many other cancer treatment schemes, patients usually tolerate antibody therapies with minimal side effects. Thus, immunotherapy with antibodies represents an interesting opportunity for combination with standard cancer therapy modalities such as chemotherapy, and for targeting drugs, radionuclides and toxins to tumor neovasculature.

As we learn more about the molecular composition of the neovasculature at different body sites and in various diseases, a considerable heterogeneity in antigen expression is discovered. This is nicely exemplified by the extra domain C of tenascin-C, which is abundantly expressed in the neovasculature of the vast majority of aggressive brain and lung tumors, but is virtually undetectable not only in normal tissues, but also in many other tumor types. Such differences in antigen abundance and distribution offer yet further possibilities for the development of selective biomedical strategies.

In general, progress in vascular tumor targeting will strongly depend on advances in the discovery of vascular tumor antigens, on the development of technologies for ligand identification, as well as on the integration of these key technologies into biopharmaceutical agents with pharmacokinetic and biological properties which are ideally suited for the tumor type to be cured.

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