Review

License for destruction: Tumor-specific cytokine targeting

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Stroma is an integral part of solid tumors and plays a key role in growth promotion and immune suppression. Most current therapies focus on destroying tumors and/or abnormal vasculature. However, evidence is emerging that anticancer efficacy improves with vessel normalization rather than destruction. Specific targeting of cytokines into tumors provides proof-of-concept that tumor stroma is dynamic and can be remodeled to increase drug access and alleviate immune suppression. Changing the inflammatory milieu 'opens' tumors for therapy and thus provides a license for destruction. This involves reprogramming of paracrine signaling networks between multiple stromal components to break the vicious cycle of angiogenesis and immune suppression. With active immunotherapy rapidly moving into the clinic, local cytokine delivery emerges as an attractive adjuvant.

Microenvironmental therapy

Tumor cells are embedded in stroma which is composed of blood vessels, immune cells, and connective tissue including fibroblasts and the extracellular matrix (ECM). Stroma is crucially involved in tumor growth, invasion, and metastasis [1,2]. The tumor microenvironment also impedes drug delivery and thus reduces the efficacy of conventional antitumor therapies such as chemotherapy and radiation therapy [3,4]; similar mechanisms contribute to a general lack of cytotoxic T cell function and antitumor immunity [5,6].

Tumors create their own microenvironments, which are diverse and tumor type- and stage-dependent; however, tumors also share common stromal features and signaling themes. In particular, intricate relationships between inflammatory factors, macrophages, and blood vessels exist in most solid tumors, which modulate growth, therapeutic response, and ultimately relapse [7–9]. Disruption of these relationships and remodeling of stroma opens tumors for cytotoxic drugs or immune destruction (see Glossary) and thus creates new and exciting opportunities for anticancer therapy. As new stromal markers and functional relationships are discovered, potential therapeutic strategies include local delivery of drugs/toxins into the tumor microenvironment via peptides or antibodies. *In vivo* screening of phage display libraries of peptides or antibodies have identified unique targets for stroma-specific molecules *in situ*, resulting in successful development of

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Glossary

Cancer immunotherapy: uses the immune system to reject tumors. Only recently, active immunotherapy has become available for cancer patients with the clinical approval of two agents: sipuleucel-T (Provenge, Dendreon), an autologous, dendritic cell (DC) vaccine for advanced prostate cancer, and Ipilimumab (Yervoy, Bristol-Myers Squibb), a monoclonal antibody to cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) for metastatic melanoma.

CD40: is a co-stimulatory molecule expressed on antigen presenting cells and in tumor stroma. CD40 agonists activate T cells and modulate tumor stroma. Antitumor effects of agonistic CD40 antibodies are currently assessed in clinical trials.

CpG–ODN: CpG–ODN is a synthetic reagent which consists of immunostimulatory oligodeoxynucleotides (ODNs) with cytosine–guanine-rich (CpG) motifs and a phosphothioate-stabilized backbone. It mimics bacterial DNA and is a potent immune adjuvant. Through its interaction with TLR-9, CpG–ODN activates B cells and plasmacytoid DCs in humans and a broader spectrum of DCs, B cells, and macrophages in mice.

Hypoxia: tumor hypoxia or low oxygen concentration is a consequence of vascular abnormalities and low oxygen supply in rapidly growing tumors which outgrow their blood supply. Anticancer drugs are often unable to penetrate into hypoxic areas.

Interstitial fluid pressure (IFP): is regulated by stromal cells and the ECM. Solid tumors have a raised IFP due to increased vessel permeability, lymphatic vessel abnormalities, and interstitial fibrosis. Increased IFP in tumors reduces perfusion and drug penetration.

Pericytes: specialized mesenchymal cells which line and stabilize endothelial cells of small capillaries. Pericytes are part of the abnormal vascular bed in tumors, are often loosely attached to tumor endothelial cells, and are reduced in numbers or are less mature. Pericyte coverage is an important parameter for the assessment of tumor vessel remodeling/normalization.

Phage display libraries: phage display libraries are bacteriophage particles with randomly displayed peptides or antibody fragments of different binding specificities. Each peptide/antibody fragment recognizes different target molecules. Libraries can be injected intravenously to specifically screen for binding activities in a particular tissue or tumor. Phages that bind to target molecules in the tumor of interest are enriched after multiple rounds of biopanning. Subsequently, binding moieties are analyzed and can be developed into targeting vehicles.

Regulator of G protein signaling 5 (RGS5): RGS5 is a member of the regulator of G protein signaling family, which is abundantly expressed in vascular smooth muscle cells and modulates vascular homeostasis by controlling G-protein-coupled receptor signaling. RGS5 is specifically upregulated in tumor pericytes. Removing RGS5 from the tumor microenvironment in murine pancreatic tumors leads to normalization of the tumor vasculature and improved response to immunotherapy.

Tumor-associated macrophages (TAM): are innate immune cells which are found in the majority of solid tumors. TAMs represent M2-activated macrophages, which promote tumor growth by secreting factors that stimulate breakdown of the ECM and vessel growth, and inhibit anticancer immunity. By contrast, M1 macrophages support antitumor immunity.

Box 1. Current tumor peptide targeting

Binding activities of peptide ligands can be based on overexpression and also on cancer-specific cellular localization of their receptors which differs from normal cells. For example, the F3 peptide, a fragment of the human high mobility group protein 2, binds to nucleolin, which can be aberrantly exposed on the surface of endothelial and tumor cells during carcinogenesis [73]. Tumor endothelial cells are surrounded by pericytes and a basement membrane, both of which are commonly altered in solid tumors. Collagen IV, for instance, is modified by matrix metalloproteinases (MMPs) during angiogenic vessel remodeling and a peptide sequence (TLTYTWS) has been identified that specifically binds to collagen IV modified by MMP-2 [74]. Peptide ligands such as CPRECES [receptor: aminopeptidase A (CD249)], CRGRRST [RGR peptide, putative receptor: platelet-derived growth factor receptor (PDGFR)β], CSRNLIDC (pBP peptide, receptor: PDGFRβ), or indeed NGR (receptor: CD13) with vascular targeting properties may also bind to tumor pericytes [54,75–78]. As a consequence of leaky tumor blood vessels, blood clotting complexes are located in tumor vessels and surrounding stroma, which provide specific docking signals for the pentapeptide CREKA [79]. Besides blood vessels, lymphatic vessels are also an integral part of solid tumors and intimately involved in metastatic spread. Peptides such as LyP-1 (CGNKRTRGC), which have been identified as ligands for the molecule p32, when expressed on the cell surface, bind to tumor-associated lymphatics, some p32-positive tumor cells, and intratumoral macrophages [70].

targeted delivery of diagnostic or bioactive fusion compounds [10–12]. These compounds may have efficacy on their own but, in the right context, can also enhance other anticancer drugs and, importantly, antitumor immunity. Here, we discuss the latest aspects of 'microenvironmental therapy', which exploits the conventional concept of peptide or antibody-mediated targeting in the context of tumor stroma modulation and vascular normalization for improved combination therapies.

Targeting tumor vasculature

The majority of peptides or antibodies with high stromal affinity identified by *in vivo* perfusion methods bind to the angiogenic vasculature in solid tumors [10,11] (Box 1). This is not surprising because tumor blood vessels are morphologically and functionally different from normal blood vessels [13]. Moreover, intravenous injection and blood circulation facilitate binding of ligands on the luminal side of endothelial cells. Such peptides, which generically home to angiogenic vessels, have the RGD (Arg–Gly–Asp) or NGR (Asn–Gly–Arg) motifs, which bind to $\alpha_v\beta_3$/$\alpha_v\beta_5$ integrins and the metalloprotease aminopeptidase N (CD13), respectively [14,15]. Most prominent vessel-targeting antibody fragments (single chain variable fragments, scFv) are directed against specific splice variants of fibronectin (L19, F8) and tenascin C (G11, F16), both part of the ECM surrounding tumor neovasculature [16–19]. Thus, these targeting reagents commonly recognize molecules actively involved in cell–cell/cell–matrix interactions and angiogenic vessel remodeling, and are frequently overexpressed in the tumor vasculature. Importantly, the value of these peptides/antibodies is in their potential to deliver therapeutic payloads into precise tumor stromal compartments.

Therapeutic tumor targeting

Peptide or antibody fusion compounds when used as carrier molecules provide a unique opportunity to improve anticancer therapy whilst reducing harmful side effects. An impressive spectrum of fusion compounds for delivery of toxic agents, radionuclides, procoagulation factors, and cytokines have been tested in preclinical tumor models, and some clinical trials are underway (Table 1). For instance, application of the NGR motif fused with proapoptotic factors such as tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) [20] or the D-amino acid peptide $_D$[KLAKLAK]$_2$ [21] induces tumor endothelial cell apoptosis. Vessel-targeted truncated tissue factor (tTF) causes thrombosis and vessel collapse in animal models and some reduction in tumor perfusion in a clinical case [22]. Chemotherapeutic drugs have also been directly conjugated to targeting moieties or used in various combination therapies [23,24]. Indeed, to date, most targeting efforts have been directed to improve cytotoxic drug delivery into tumors and enhance drug penetration into parenchyma to amplify antitumor cytotoxicity (Box 2).

Inflammatory factors such as cytokines represent attractive compounds for specific, high-dose delivery into tumors. Antitumor effects of cytokines have been well documented over the past decades. In particular high-dose TNFα disrupts angiogenic vessels and is currently used in isolated limb perfusion to treat locally advanced melanoma and soft tissue sarcoma [25,26]. However, the clinical application of cytokines has been restricted to local treatment due to high toxicity. Precise targeting of tumor vessels is therefore a promising strategy and has been employed for various peptide/antibody–cytokine chimeric compounds, which include interleukin (IL) 2, IL12, interferon γ (IFNγ), and TNFα (Table 1). Most cytokines are not

Box 2. Vascular homing peptides: optimizing cytotoxic antitumor effects

One strategy to improve vascular targeting and antitumor effects is to use nanoparticles decorated with multiple vascular homing peptides and loaded with cytotoxic drugs for release at the tumor site [11]. Multivalent targeting of cytotoxic nanocarriers can overcome some pharmacological limitations of directly conjugated peptide ligands. However, access into tumors beyond the vasculature remains challenging. Recently, Ruoslahti and colleagues described a series of peptides which specifically bind to tumor stroma and also penetrate into tumor tissue [94–96]. Most remarkable is the capacity of prototypic iRGD peptide (CRGDK/RGPD/EC) to deliver payloads such as doxorubicin into tumors simply by coadministration, thus abolishing the need to produce fusion compounds. This approach results in a 14-fold increase in doxorubicin containing liposomes in tumors compared with injection of doxorubicin liposomes without iRGD and significantly enhances antitumor effects [94]. Another recent development elegantly harnesses biological effector cascades to combine traditional chemotherapy with vascular destruction. For instance, rod-shaped gold nanoparticles (nanorods) which passively home into tumors are used to induce coagulation under near-infrared light irradiation. This in turn significantly enhances accumulation of doxorubicin-loaded liposomes conjugated with the peptide substrate for the coagulation factor FXIII in human breast cancer xenotransplants [97]. Specific targeting of coagulated tumor vessels for delivery of cytotoxic drugs may overcome some limitations arising with antiangiogenic therapy such as decreased tumor perfusion and limited drug access.