Targeting of drugs and nanoparticles to tumors

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The various types of cells that comprise the tumor mass all carry molecular markers that are not expressed or are expressed at much lower levels in normal cells. These differentially expressed molecules can be used as docking sites to concentrate drug conjugates and nanoparticles at tumors. Specific markers in tumor vessels are particularly well suited for targeting because molecules at the surface of blood vessels are readily accessible to circulating compounds. The increased concentration of a drug in the site of disease made possible by targeted delivery can be used to increase efficacy, reduce side effects, or achieve some of both. We review the recent advances in this delivery approach with a focus on the use of molecular markers of tumor vasculature as the primary target and nanoparticles as the delivery vehicle.

Introduction

The concept of targeted drug delivery is attractive because it recapitulates some of the advantages of topical application of drugs: high local concentration and low systemic exposure. In practice, this approach has met with some success but has not provided the hoped-for "silver bullets." However, recent developments in the field have rekindled interest in the targeting approach. We call this mode of drug delivery "synaphic" targeting; it is also referred to as pathotropic or active targeting. Cancer stands out as a disease most likely to benefit from targeted drug delivery. Tumor cells express many molecules on their surface that distinguish them from normal cells. Traditionally, such molecules were detected with antibodies, but screening of peptide and aptamer libraries has greatly expanded the number of tools available for selective binding to tumor cells (for reviews see Ruoslahti, 2002; Peer et al., 2007). Leukemia and lymphoma treatments with antibodies conjugated to a radioisotope have been in clinical use for several years (Sharkey and Goldenberg, 2005). However, this approach has not been as successful with solid tumors. The apparent reason is the difficulty in delivering drugs into these tumors; drugs only penetrate a few cell diameters into the extravascular tumor tissue from blood vessels (Hambley and Hait, 2009). This low penetration appears to arise from two main factors: first, tumor vessels are poorly perfused with blood and are dysfunctional, which limits the delivery of blood-borne compounds to tumors (Jain, 1999). Second, tumors have a high interstitial pressure thought to result from dysfunctional lymphatics, which causes tissue fluid to flow out of the tumor, working against diffusion of drugs from the blood vessels into the tumor (Jain, 1999; Heldin et al., 2004). The leakiness of tumor vessels partially makes up for the poor penetration (the so-called enhanced permeability and retention [EPR] effect), but EPR is not very effective, and its size dependency and variability from tumor to tumor limit its usefulness (Maeda et al., 2000; Iyer et al., 2006; Sugahara et al., 2009). Interstitial fibrosis can further retard the diffusion of compounds through tumors (Olive et al., 2009). Targeting treatments to selective markers in tumor vessels does not suffer from some of these drawbacks of targeting tumor cells; in particular, no tissue penetration is required for the compound to reach its target. The luminal side of tumor vessels is fully accessible to compounds circulating in the blood, and the vessels can serve as a gateway to the tumor interior for compounds concentrated in the vessels. Using a targeting probe with tumor-penetrating properties and a receptor that is shared between tumor vessels and tumor cells provides additional advantages (Fig. 1). Thus, we have chosen to focus this review on targeting approaches that make use of specific markers in tumor vessels. We will also discuss solutions to the poor penetration of compounds into tumor tissue and the roles that nanoparticles can play in targeted therapies.

Molecular signatures in tumor vessels

Distinct features of tumor vessels. Tumor blood vessels are distinct from normal vessels. In addition to being tortuous, uneven in diameter, and leaky, tumor vessels express

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Abbreviations used in this paper: CendR, C-end rule; EPR, enhanced permeability and retention; MPS, mononuclear phagocyte system; RES, reticuloendothelial system; TEM, tumor endothelial marker.

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 188 No. 6 759–768
www.jcb.org/cgi/doi/10.1083/jcb.200910104

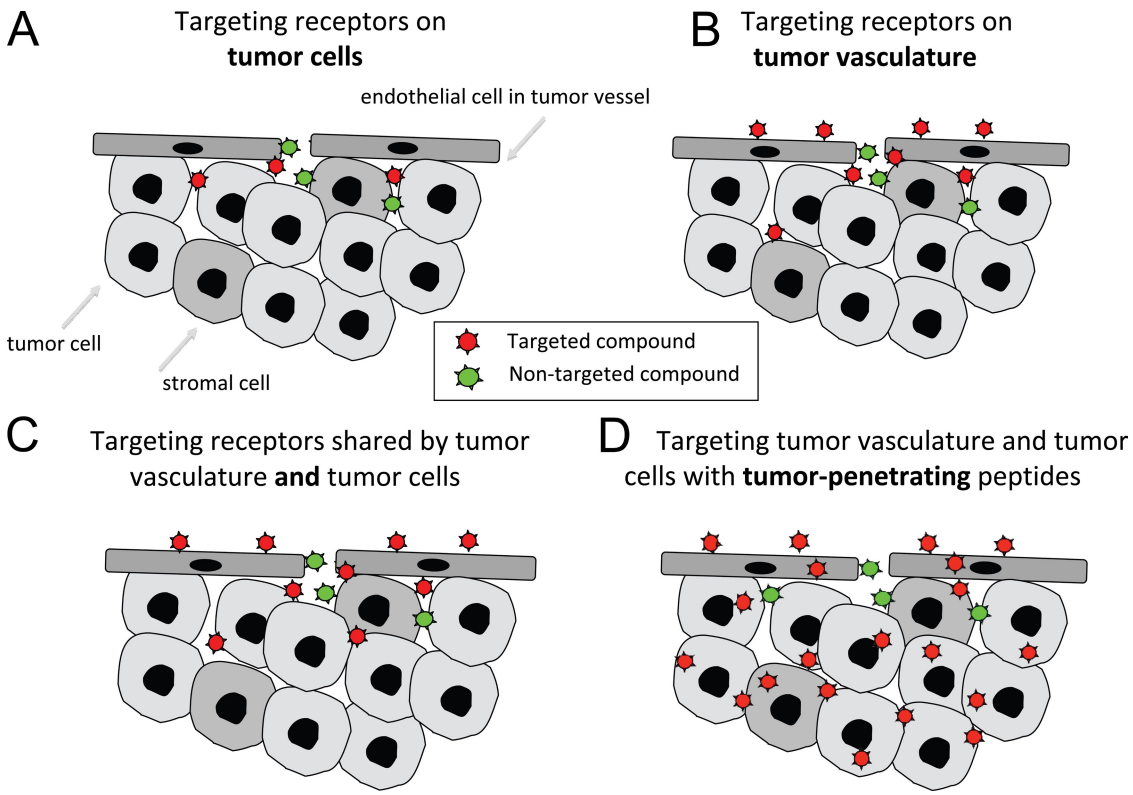

Figure 1. **Synaphic targeting of tumors.** The targeted receptors can be on tumor cells, tumor vessels, or shared by both. (A) Probes that recognize solely tumor cells provide little improvement of tumor accumulation over a nontargeted probe. (B) Probes that recognize tumor vessels accumulate in the tumor, but entry into tumor tissue relies on passive mechanisms. (C) Probes that recognize both the vessels and tumor cells combine the (limited) efficiency of the two targeting mechanisms. (D) Tumor-penetrating targeting probes (so far only peptides with such characteristics are known) provide a particularly potent targeting system.

various cell surface and extracellular matrix proteins that normal vessels do not express or do so at much lower levels than tumor vessels (for review see Ruoslahti, 2002). The expression of many of these proteins in tumor vessels is associated with angiogenesis, and they are often functionally important in that process (Hanahan and Folkman, 1996; Alitalo and Carmeliet, 2002). Tumors also contain lymphatic vessels, and many tumors produce growth factors that stimulate lymphangiogenesis (Karpanen and Alitalo, 2008). Lymphatics are not necessary for tumor growth but are important conduits of metastasis. Like tumor blood vessels, tumor lymphatics can also express specific molecular markers.

Screening for markers in tumor vasculature. Screening of phage-displayed peptide libraries, particularly when performed in vivo, has provided a very useful discovery tool for vascular markers in tumor vessels and elsewhere (Pasqualini and Ruoslahti, 1996). A major advantage of the in vivo phage screening is that it is unbiased in revealing what works in vivo. Other unbiased methods, such as antibody-based screens (Jacobson et al., 1996), cloning strategies (Carson-Walter et al., 2001), and in vivo biotinylation (Borgia et al., 2010), have also been used successfully in analyzing tumor vasculature. Phage screening has uncovered a large number of tumor-homing peptides that have been used to identify the corresponding binding protein (receptor). An early study on tumor-homing peptides (Arap et al., 1998) validated the method by producing tumor-homing peptides with RGD (arginine/glycine/aspartice acid) and NGR (asparagine/glycine/arginine) motifs, which had been previously identified in screens for integrin-binding peptides performed in vitro. F3 is an example of a novel tumor-homing peptide identified by in vivo phage screening (Porkka et al., 2002). F3 binds to nucleolin, which is ubiquitous as an intracellular protein but is expressed at the cell surface of endothelial cells and tumor cells in vivo (Christian et al., 2003). In vitro, all cells seem to be positive for cell surface nucleolin (Borer et al., 1989; Bonnet et al., 1996) presumably because cultured cells resemble cells that have been activated in vivo. Cell surface nucleolin is an angiogenesis marker that is both a suitable target for drug delivery (Christian et al., 2001; Reddy et al., 2006; Henke et al., 2008; Drecoll et al., 2009) and involved in the angiogenesis process (Fogal et al., 2009). The in vivo screening of phage libraries has also produced several potent tumor-homing peptides, the target molecules of which remain to be identified (Hoffman et al., 2003; Joyce et al., 2003; Järvinen and Ruoslahti, 2007; Chang et al., 2009).

The expression of intracellular proteins such as nucleolin at the cell surface of tumor cells and tumor endothelial cells appears to be a general principle. Phage display is particularly well suited for the discovery of such markers because the method inherently relies on binding to accessible targets on the cell surface rather than overall expression levels. In vivo cell surface labeling followed by monoclonal antibody production

and proteomics analyses is another way of interrogating the cell surface. In addition to aforementioned nucleolin, the cytoplasmic proteins annexin1 (Oh et al., 2004) and plectin-1 (Kelly et al., 2008) have been found to be present at the cell surface of endothelial cells in tumors but not in normal tissues. Another example is p32 protein (gC1q receptor, hyaluronic acid–binding protein). This protein is primarily a mitochondrial protein, but it is also expressed at the cell surface of lymphatic, myeloid, and cancer cells in tumors but not in normal tissues (Fogal et al., 2008). This protein is the receptor for the tumor-homing peptide LyP-1, originally discovered using in vivo phage display (Laakkonen et al., 2002).

Adhesion receptors as angiogenesis markers. Some of the molecular markers in tumor vasculature have been found by studying the expression of known cell surface receptors in tumor vessels. A prime example is the overexpression of αvβ3 and αvβ5 integrins in angiogenic vessels (Brooks et al., 1994; Erdreich-Epstein et al., 2000; Desgrosellier and Cheresh, 2010). These integrins are prime targets for synaphic drug delivery. Vascular markers expressed on the surface of the endothelium, such as the integrins, are most readily available for the binding of blood-borne compounds. However, the ECM also contains distinct markers that can be used in tumor targeting. An alternatively spliced form of fibronectin containing an additional type III domain, ED-B, is selectively expressed in tumor (and other) angiogenic vessels (Nilsson et al., 2001). Antibodies to ED-B have been used to construct immunotoxins and other compounds for tumor targeting. Proteolytically processed type IV collagen is another matrix component that can be detected with antibodies or peptides (Roth et al., 2006; Mueller et al., 2009). The support cells (mural cells) in the vascular wall also contain markers that are specific for tumor vessels and that can be potentially useful in tumor targeting. NG2, a membrane-spanning chondroitin sulfate proteoglycan, is a cell surface marker of pericytes (and smooth muscle cells) in angiogenic vessels not expressed in the pericytes of normal vessels (Stallcup and Huang, 2008). One of the PDFG receptors is another marker that is expressed at high levels in pericytes (Song et al., 2005).

Fibrin–fibronectin complexes in tumors. Peptides that specifically bind to fibrin–fibronectin complexes or other proteins associated with these complexes also home to tumors. The walls of tumor vessels and the interstitial spaces in tumors contain products of blood clotting, presumably as a result of plasma protein seepage from leaky tumor vessels. Leaked fibrinogen is converted to a fibrin meshwork by tissue-procoagulant proteins such as tissue factor (Dvorak et al., 1985; Abe et al., 1999; Pilch et al., 2006). Other plasma proteins, plasma fibronectin in particular, become covalently linked or otherwise bound to the fibrin meshwork. These fibrin–fibronectin complexes in the walls of tumor vessels and in the tumor interstitial stroma can be accessed with peptides derived from phage screening, such as the nine–amino acid cyclic peptide CLT-1 (Pilch et al., 2006; Ye et al., 2008) and the pentapeptide CREKA (Simberg et al., 2007). Fibrin-binding peptides isolated for the purpose of targeting blood clots in cardiovascular disease would presumably behave similarly if tested for tumor homing. The CREKA peptide has been used to confer a new function to nanoparticles: self-amplification of tumor homing (see Amplified tumor homing; Simberg et al., 2007).

Table I. **Cell surface and ECM-docking receptors in tumor vessels**

Receptor	References
RGD-directed integrins (αvβ3 and αvβ5)	Ruoslahti, 2002; Desgrosellier and Cheresh, 2010
Aminopeptidase N	Pasqualini et al., 2000
TEMs	Carson-Walter et al., 2001
Endosialin	Christian et al., 2001
Cell surface nucleolin	Christian et al., 2003
Cell surface annexin-1	Oh et al., 2004
Cell surface p32/gC1q receptor	Fogal et al., 2008
Cell surface plectin-1	Kelly et al., 2008
Fibronectin ED-B	Nilsson et al., 2001
Fibrin–fibronectin complexes	Pilch et al., 2006; Simberg et al., 2007
Interleukin-11 receptor α	Lewis et al., 2009
Protease-cleaved collagen IV	Xu et al., 2001; Mueller et al., 2009

Tumor endothelial markers (TEMs). Surveying mRNA expression by the serial analysis of gene expression technique has revealed a large number of striking differences between endothelial cells isolated from human colon cancers and those from adjacent normal tissue (Carson-Walter et al., 2001). Among these TEMs are collagens, some of which are expressed at strikingly high levels in tumor endothelial cells, at least at the mRNA level. The high collagen expression may relate to the extensive fibrosis found in many tumors and recently shown to contribute to the poor penetration of drugs into tumors (Olive et al., 2009). Perhaps the most interesting among the TEMs is TEM 8, which is one of the two receptors for the anthrax toxin (Nanda et al., 2004). An effort is under way to develop anthrax toxin variants that bind only to TEM 8 and that could be used to target tumor vasculature for destruction. Table I provides a list of the principal cell surface markers available in tumor vessels for docking-based targeting.

Stage-specific markers. The molecular angiogenesis signatures vary depending on the state of tumor development. Initiation of angiogenesis (the angiogenic switch) occurs already in premalignant lesions (Hanahan and Folkman, 1996). Peptide probes that distinguish between the blood vessels of premalignant and fully malignant lesions of some de novo cancers in mice have been reported (Hoffman et al., 2003; Joyce et al., 2003). The vascular molecules recognized by these peptides remain to be identified. It may also be possible to develop targeting probes that distinguish between physiological and tumor angiogenesis (Seaman et al., 2007).

Nontumor angiogenesis. A significant issue in the use of angiogenesis-detecting probes in cancer diagnosis or therapy is that angiogenesis also occurs in regenerating tissues and in inflammation. This poses a potential problem for tumor targeting, as angiogenesis associated with tissue repair in conditions coexisting with cancer, such as myocardial infarction or stroke, could be inadvertently targeted for destruction. This circumstance emphasizes the need to discover vascular

markers with more focused target recognition properties. Probes that recognize tumor type–specific markers would fall into this category, as they obviously could not be targeting all forms of angiogenesis. The idea that this level of specificity can be achieved with tumor vessels has been demonstrated with tumor type–specific peptides (Hoffman et al., 2003; Joyce et al., 2003; Laakkonen et al., 2004). Peptides like this should make diagnostic and therapeutic applications possible that are more selective than angiogenesis-based targeting. The use of peptides (or other types of probes) with this kind of focused specificity would likely require first diagnostically assessing the tumor of each individual patient for the selective expression of the appropriate receptor. Such personalized medicine seems certain to become increasingly prevalent.

Functional role of tumor vessel markers. The αv integrins play an important role in angiogenesis, although the details of their involvement in this process remain to be fully elucidated (Desgrosellier and Cheresh, 2010). Peptides containing an NGR sequence motif had previously been shown to bind weakly to the RGD-binding site of integrins (Koivunen et al., 1994), but this motif was later identified as the binding motif in tumor-homing peptides that were more potent than could be expected on the basis of the weak integrin binding (Arap et al., 1998). It was subsequently shown that the NGR peptides recognize aminopeptidase N (Pasqualini et al., 2000) and potentially, after a chemical alteration, αv integrins (Curnis et al., 2008). Like nucleolin, aminopeptidase N is functionally important in the angiogenesis process (Pasqualini et al., 2000; Rangel et al., 2007). These findings serve as a paradigm to illustrate a discovery process in which a new homing peptide is discovered in phage screening, the receptor for the peptide is identified by biochemical methods such as affinity chromatography, and subsequent studies reveal a role for the receptor in the biology of tumor vessels. Once the receptor is identified, an effective therapy may be engineered. Both F3 and the NGR motif peptides have been used to target drugs to tumors (Curnis et al., 2004; Reddy et al., 2006; Henke et al., 2008), and aptamers that bind nucleolin are being pursued in phase I clinical trials (Laber, D., V.R. Sharma, D.A. Laber, V.R. Sharma, L. Bhupalam, B. Taft, F.J. Hendler, and K.M. Barnhart. 2005. American Society of Clinical Oncology Annual Meeting Proceedings. Abstr. 3064).

Delivery of therapeutic agents to vascular targets

Targeting integrins. The αvβ3 and αvβ5 integrins are highly expressed in tumor endothelium, and their level of expression may be highest in the vessels of the most malignant tumors (Erdreich-Epstein et al., 2000). Enhanced drug delivery with vascular homing peptides has been accomplished using a cyclic peptide containing the integrin-binding RGD motif (CRGDC) to deliver doxorubicin to tumors (Arap et al., 1998).

Remarkable success in targeting the cytokine TNF into tumors has been reported with RGD and NGR peptides; the targeted cytokine was effective in doses as much as 1,000-fold lower than the usual dose and effectively mitigating side effects as a result of the high toxicity of this cytokine (Curnis et al., 2004). These same peptides have also been used to deliver tissue factor to induce blood clotting specifically in tumor blood vessels, with resulting occlusion of the vessels and tumor necrosis (Bieker et al., 2009). A targeted TNF is currently in clinical trials (Paoloni et al. 2009; Gregorc et al., 2010).

Conjugates of an antibacterial peptide, which destroys mitochondria in mammalian cells causing apoptosis, with either the RGD or NGR peptide also inhibited tumor growth in mice, whereas either peptide alone was inactive (Ellerby et al., 1999). Moreover, targeting the same proapoptotic peptide to the blood vessels of the normal prostate caused partial destruction of the prostate and delayed the development of cancers in transgenic prostate cancer mice (Arap et al., 2002). The potential of synaphic targeting is very well illustrated; the combination of homing peptides with nonselectively toxic compounds, such as proapoptotic peptides and TNF, can profoundly alter the in vivo activity of the toxins. RGD peptides and antibodies to αvβ3 integrin have also been successfully used in targeted delivery of diagnostic probes to tumors (Sipkins et al., 1998; Stollman et al., 2009; Sugahara et al., 2009), and imaging probes based on this approach are in clinical trials. Drug-loaded nanoparticles have also been targeted with RGD peptides to suppress tumor growth or metastasis (Hood et al., 2002; Murphy et al., 2008; Sugahara et al., 2009). Finally, RGD and other tumor-homing peptides have been used to alter the host range of viral gene therapy vectors (Wickham, 2000; Haviv et al., 2002). Several homing peptides that bind to receptors other than integrins have also been successfully used in preclinical studies to target gene therapy vectors, drugs, and biologicals into tumors (Müller et al., 2003; Hamzah et al., 2008; Chang et al., 2009; Karmali et al., 2009).

Tumor-penetrating peptides. A major problem with many of the currently used tumor-targeting probes is that a reagent directed to tumor cells will be impeded by the poor permeability of tumors to blood-borne compounds. This problem is particularly prominent with solid tumors, which have a high interstitial pressure, presumably because their blood vessels tend to be leaky and their lymphatic vessels poorly functional (Jain, 1999). Drugs generally do not penetrate further than three to five cell diameters from blood vessels, which leaves more distantly located tumor cells without any drug or exposes them to low drug concentrations that are likely to facilitate the development of resistance (Hambley and Hait, 2009). Despite these limitations, a homing peptide that binds to the Her2 receptor (Gee et al., 2008) has been used to deliver compounds to tumors that overexpress this receptor. Folic acid is another probe commonly used to target the folate receptor, which is overexpressed by tumor cells in many tumors (for review see Salazar and Ratnam, 2007). Experimental and theoretical results indicate that this increase in efficacy is not dominated by changes in overall drug uptake by the tumor (i.e., increased volumetric concentration) but rather changes in cellular internalization of the drug or how long it is retained in the tumor (Bartlett et al., 2007). Thus, there is little or no specific accumulation of probes targeted solely to tumor cells (Fig. 1 A). Results with nanoparticles targeted to tumor cells should be interpreted with particular care. Nanoparticles are small (from a few to

200 nm in diameter) particles that can serve as drug carriers and contrast agents (for imaging) in medicine. Although small in comparison with cells, nanoparticles are much larger than molecules and are less likely to penetrate the vascular wall and gain access to tumor cells than small molecular mass drugs or even antibodies. Tumor blood vessels are more readily available for targeting than the tumor cells and can mediate specific targeting (Fig. 1 B). It is not clear to what extent the Her2 or folate receptors might be expressed on tumor endothelial cells, where they would contribute to the uptake of drugs by the tumor. Some other receptors used in synaphic targeting are expressed both in the tumor vessels and on tumor cells. Examples include αv integrins and nucleolin. These dual targeters are more effective than probes that recognize only the vessels or the tumor cells (Fig. 1 C). However, strategies to increase overall tumor accumulation of drugs and nanoparticles are still needed. Targeting with tumor-penetrating peptides, particularly when the peptide binds both to the tumor endothelium and the tumor cells, provides such a strategy (Fig. 1 D).

The laboratory of E. Ruoslahti has recently discovered a tissue–cell penetration system that makes it possible to derive peptides that not only home to a specific target tissue but also penetrate into that tissue. The peptides contain a tissue penetration motif, R/KXXR/K, which has to be exposed at the C terminus of a peptide (or protein) to be active (the C-end rule [CendR]; Teesalu et al., 2009). A tumor-homing CendR peptide contains both a tumor-specific homing sequence and a cryptic (not C terminal) CendR sequence. The homing sequence takes the peptide to the vascular endothelium in the target tissue, where the peptide is proteolytically processed by an endogenous protease such that the CendR motif becomes C terminal and active. The activated CendR motif then binds to a different receptor (neuropilin-1), which mediates extravasation, tissue penetration, and cell entry of the C-terminally truncated peptide and any payload attached to it. An RGD containing a CendR motif, iRGD, exemplifies the capabilities of these peptides. The iRGD peptide penetrates into tumor tissue and is capable of carrying 10 times more drug cargo into a tumor than a conventional RGD peptide (Sugahara et al., 2009).

Several aforementioned homing peptides may be tumor-penetrating peptides similar to iRGD. F3 (Porkka et al., 2002), LyP-1 (Laakkonen et al., 2002), and CRGRRST (Joyce et al., 2003) each contain a potential CendR sequence. Moreover, F3 and LyP-1 have been shown to cause extravasation of their cargo, which can be as large as a nanoparticle, with subsequent uptake into tumor endothelial cells and tumor cells (Porkka et al., 2002; Laakkonen et al., 2004; Karmali et al., 2009). Coating of abraxane, which is a nanoparticle drug composed of paclitaxel and albumin, with the LyP-1 or iRGD peptide made the drug capable of penetrating into tumor tissue, resulting in several-fold higher activity than that of the original drug (Karmali et al., 2009; Sugahara et al., 2009). The tissue-penetrating properties of these peptides and their internalization into cells makes them particularly efficient in achieving a high concentration of the peptide and any payload attached to it in tumor tissue. Unlike the cell-penetrating peptides related to the human immunodeficiency virus Tat protein, which do not display any cell type specificity (Gump and Dowdy, 2007), the CendR tumor-homing peptides are tumor specific. Jiang et al. (2004) have described a peptide design in which a negatively charged sequence tethered to a cationic cell-penetrating peptide blocks the cell-penetrating activity until a tumor protease cleaves the tether. The authors achieved a threefold increase in tumor homing. The greater tumor-homing selectivity of peptides derived from in vivo phage display such as iRGD (12-fold) is likely because of the presence of a homing sequence (RGD in iRGD) in these peptides (Sugahara et al., 2009).

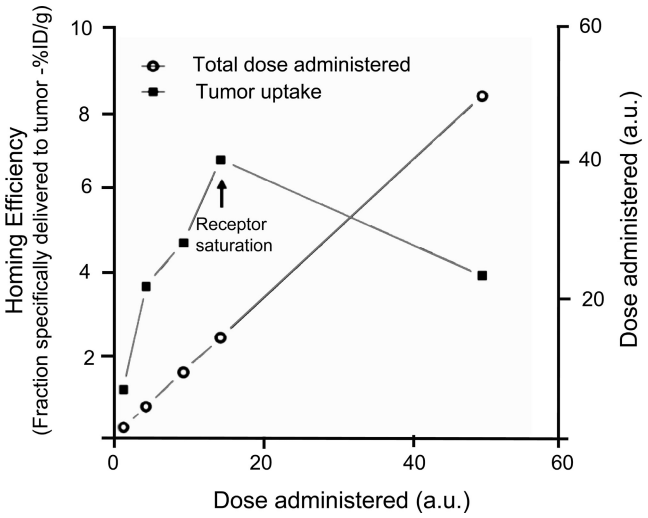

Figure 2. **Saturation of receptors affects the specificity of synaphic targeting.** Once the receptors of the homing peptide have been saturated, the specificity of the targeting declines (adapted from experimental data in Kranenborg et al., 1998). au, arbitrary units.

Limitations of synaphic targeting

Receptor capacity. An important factor to consider in synaphic tumor targeting is the capacity of the receptors that are targeted by the probe. The number of cell surface receptors and their availability determine how many molecules of a targeting compound can be specifically bound at the tumor site. Under ideal conditions (infinite binding affinity), the amount of compound that can be bound by the tumor equals the number of available receptors (assuming a 1:1 binding ratio and negligible turnover). For example, assuming a tumor cell volume of one nanoliter and the presence of 100,000 receptors per cell, there would maximally only be a total of 0.166 nmol of receptor per gram of tumor. The binding of the targeting ligand for the receptor is likely to have a high nanomolar to low micromolar disassociation constant, which means that more of the targeting compound has to be administered than can be accommodated by the receptors to drive the interaction toward binding and receptor saturation. Moreover, only a fraction of the receptors is likely to be available to bind a ligand introduced into the blood stream. Any excess of the targeting compound is going to be handled by the body like any other nontargeted compound. If the amount of free targeting compound substantially exceeds the receptor-bound amount, the effect of specific targeting will be drowned out by nonspecific background. This circumstance, illustrated in Fig. 2, is underappreciated in the

field, despite repeated demonstrations that targeted compounds are more differentially active when administered at low doses. One potential solution to this problem is to use higher affinity ligands for the targeting, but this strong binding can lead to reduced tumor penetration through the so-called binding site barrier (van Osdol et al., 1991; Thurber et al., 2008). Other potential solutions include using anticancer agents with higher potency than most current drugs, using nanoparticle delivery vehicles that deliver more drug per receptor occupied than one to one conjugates, or inducing more binding sites in the tumor (see Amplified tumor homing).

Monovalent versus multivalent binding. Low affinity of a ligand for its receptor can seriously limit the targeting efficiency or even make it unachievable. Making the low affinity ligand multivalent can circumvent this problem. Multiple weak interactions produce strong binding. Many natural processes, such as antibody interactions, rely on this principle. For example, because each of the six binding sites in IgM antibodies is generally of low affinity, IgM antibodies rely on multivalent binding. Cells adhere through multivalent interactions between integrins and adhesion proteins such as fibronectin. Phage display with cells in vitro or tissues in vivo as the target (Hoffman et al., 2003) is a prime example of a system that probes this moderate affinity, multivalent landscape. Thus, phage display complements other target discovery methods such as those based on antibodies (Jacobson et al., 1996; Oh et al., 2004) or cloning methods (Seaman et al., 2007). The enhanced avidity from multivalency is usually the result of an unaffected binding rate (on rate [k_{on}]) but a reduction in off rate (k_{off}) for the multiple interactions. Multivalency is important in nanoparticle-based targeting because nanoparticles generally carry more than one targeting ligand and are therefore capable of multivalent binding. This concept is particularly relevant for peptides, which typically bind to their targets with relatively modest (low micromolar) affinities. Reulen et al. (2009) converted a nonbinding variant of a collagen-binding protein into an active targeting probe by inserting multiple copies of the protein into a micelle, artificially producing a multivalent ensemble. The reported enhancements from multivalency are as large as 10^8 but are more typically 10–10^4. Interestingly, as few as four RGD peptides could provide a 25-fold enhancement in binding of a 30-nm particle to endothelial cells, and just three folate groups led to a 2,500-fold enhancement in dendrimer binding to a surface (dendrimers are branched synthetic polymers that can form nanoparticles and present ligands in a multivalent fashion in which the valency can be readily controlled; Montet et al., 2006; Hong et al., 2007). At the same time, multivalent peptide presentation can increase recognition of nanoparticles by the reticuloendothelial system (RES; also known as the mononuclear phagocyte system [MPS]).

Multivalency may partially explain the remarkable 1,000-fold increase in the antitumor activity of TNF observed when homing peptides recognizing tumor vessels were added to the protein (Curnis et al., 2004). TNF is a trimeric protein, which would render the homing peptide multivalent. Another factor in the increase of activity may be that the chimeric compound will presumably engage both the homing peptide and TNF receptors and that this may result in synergistic binding. It will be interesting to see whether other antitumor compounds with their own cell surface receptor, such as the Her2 antibody trastuzumab, would also benefit from homing peptide targeting in this manner.

The stability of targeting probes. Elimination from the circulation and degradation are among the main factors that determine the efficiency of a targeting probe. Short in vivo half-life can be an advantage in imaging because it quickly eliminates the background caused by excess probe. However, in drug targeting, short half-life gives the targeted drug less time to penetrate into the target tissue. Long circulation times are especially important when the target is outside the vasculature, although tumor-penetrating peptides offer a potential solution to this problem (Sugahara et al., 2009). The half-life primarily depends on the rate of elimination into the urine (small molecules) and uptake by the RES in the liver and spleen (particles). Coupling a small molecular mass drug or probe to polyethylene glycol is commonly used to increase molecular mass above the kidney filtration cut-off size of 5 nm (Choi et al., 2007). Polyethylene glycol coating is also a strategy used to minimize elimination of protein therapeutics. Preventing RES uptake is particularly important when nanoparticles are used in drug delivery.

RES, which is also known as MPS, resides primarily in the liver, spleen, and lymph nodes. It eliminates foreign materials, particularly particles, including synthetic nanoparticles, from the circulation. Tumor-responsive, cleavable stealth coatings have been used to mitigate this problem (Harris et al., 2008), but the RES/MPS uptake of nanoparticles remains a major problem in the use of nanoparticles in nanomedicine. It limits the amount of drug or probe that can reach the intended target, obscures the imaging of the liver and the organs near it, and is a source of potential liver toxicity. Coating of nanoparticles with plasma proteins that mediate binding to Kupffer cell receptors in the liver is thought to underlie this phenomenon, but the unfortunate fact is that the molecular mechanisms of the uptake of nanoparticles by the RES are not really understood (for review see Moghimi et al., 2001). It has been empirically shown that particle charge (anionic or neutral), size (<100 nm), and ability to prevent complement binding can reduce rates of RES uptake and extend circulation time in mice (for review see Peer et al., 2007). Other results suggest that the RES uptake may have little to do with plasma protein–mediated opsonization (Simberg et al., 2009). The likely explanation for why this has been such an intractable problem is that the Kupffer cell receptors use multiple low affinity interactions to capture nano (and micro)-particles, rendering conventional receptor identification methods impotent in addressing this issue. The current stealth technologies to make nanoparticles unrecognizable by the RES only delay the inevitable uptake by this system. It is of major importance to nanomedicine that efficient ways of prolonging nanoparticle circulation be discovered.

Targeted delivery of nanoparticles

Regardless of the limitations of nanoparticles, nanoparticle technology offers an exciting platform for drug delivery: they can

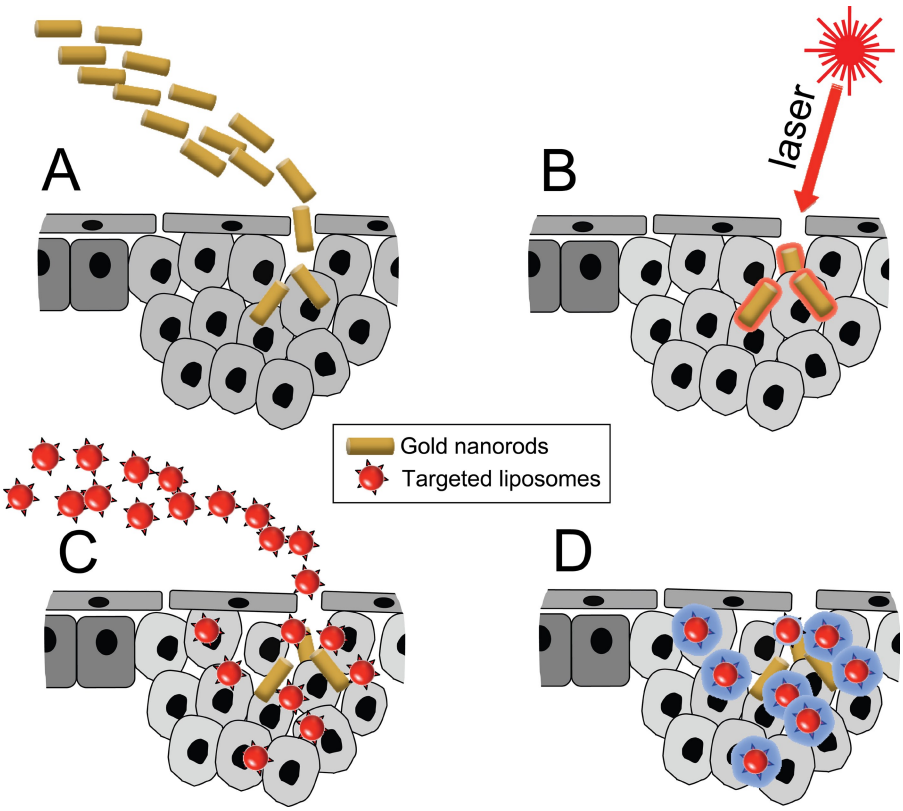

Figure 3. **Treating tumors with cooperative nanoparticles.** This scheme illustrates a method to induce cooperative nanoparticle behavior that results in more effective delivery of treatments to tumors. This example uses a two-component system consisting of gold nanorods and targeted, thermally sensitive liposomes. (A) Passive accumulation of gold nanorods. The circulating nanorods passively accumulate in the tumor as a result of leakiness of the tumor vasculature (the EPR effect). (B) Laser irradiation of nanorods activates tumor cells. The gold nanorods absorb laser energy, heating the surrounding tissue. This localized rise in temperature increases tissue permeability and induces expression of receptor proteins on the surface of the tumor cells. (C) Targeted nanoparticles (liposomes) bind to tumor. Receptor-specific targeting peptides attached onto the secondary nanoparticles allow these particles to bind to the overexpressed receptor proteins on the heat-activated tumor cells. (D) Activation of targeted liposomes releases drug. In this example, thermally responsive liposomes containing a drug payload are heated with a second laser pulse, inducing rupture of the liposome shell and release of its contents.

incorporate unique functions that cannot be engineered into simple drugs. Although both drugs and nanoparticles can be targeted to a tumor, nanoparticles can be engineered to perform more complex, cooperative targeting functions. We will next discuss self-amplified homing of nanoparticles and amplification of the targeting by nanoparticle combinations.

Amplified tumor homing. We have made use of a peptide that binds to fibrin–fibronectin complexes in blood clots to design a nanoparticle that self-amplifies its own homing to tumors. Iron oxide nanoparticles coated with the CREKA peptide bind and accumulate in tumor vessels where they cause additional clotting (Simberg et al., 2007). The approach is similar to clotting induced in tumor vessels by tumor-targeted tissue factor (Huang et al., 1997; Bieker et al., 2009) with the exception that the CREKA system is based on self-amplified nanoparticle homing. The clotting induced by the CREKA-coated iron oxide particles creates more binding sites for the peptide, which causes more clotting and so on. The 20% occlusion of tumor vessels initially obtained greatly improved tumor imaging. Recent modifications in the system have increased the occlusion rate to 60–70%, producing highly significant inhibition of tumor growth (Agemy, L., K.N. Sugahara, V.R. Kotamraju, K. Gujraty, C. Aleman, R. Nussinov, and E. Ruoslahti. 2009. Proceedings of the 100th Annual Meeting of the American Association for Cancer Research. Abstr. 3668). Importantly, although the CREKA nanoparticles are nonspecifically taken up in the RES, no clotting was seen in the vessels of the RES organs (or any other normal organs), indicating that the clotting mechanism active in this self-amplifying targeting system requires the tumor environment. As this system at this point only leverages the inherent properties of the targeted nanoparticles, it could be further engineered to carry a drug.

The ability of one structural type to perform multiple medical diagnostic or therapeutic functions is an advantageous characteristic of nanomaterials that cannot be achieved with organic small molecules. However, nanosystems that integrate multiple functions into a single structure can display reduced efficacy of the separate functions because of space and surface chemistry limitations and increased susceptibility to phagocyte uptake. Engineering separate nanomaterials that synergistically cooperate in their functions, such as tumor homing, is a way of dealing with this problem. This approach is particularly advantageous in combination therapies, which are commonly used in cancer treatments

As was discussed earlier, it would be advantageous to create more binding sites for targeted delivery in a tumor, particularly if they are within the vascular space. We recently constructed a system that leverages a biological cascade in vivo to increase the available binding sites for targeted delivery. Plasmonic nanomaterials, such as gold nanorods, present exciting opportunities for such targeting combinations. These materials are metallic structures that efficiently convert optical radiation into heat by coupling into one or more plasmon modes (Hirsch et al., 2003; Hu et al., 2006). We have recently shown that photothermal heating mediated by tumor-targeted gold nanorods can increase binding sites for targeted delivery with thermosensitive drug carriers (Fig. 3; von Maltzahn et al., 2009; Park et al., 2010). Other biological cascades, such as the protease activity that activates CendR peptides in tumors

(Sugahara et al., 2009), can be exploited. Imaging of tumors provides another good example of where the combined properties of tumor-targeted nanodevices can potentially improve the treatment of cancer patients. A tumor-targeting nanosystem that possesses both superparamagnetic and fluorescent quantum dot domains offers the possibility to provide a low resolution anatomical reference to guide the surgical procedure (by magnetic resonance imaging) and a high resolution mapping that can be visualized during surgery to identify surgical margins (by fluorescence imaging of quantum dots; Wang et al., 2004; Kim et al., 2005; Sathe et al., 2006; Kim and Taton, 2007; Song et al., 2007; Park et al., 2008; Ye et al., 2008). Recently, we have also designed iron oxide nanoparticles (nanoworms; Park et al., 2009b) with improved properties and nontoxic silicon-based quantum dots (Park et al., 2009a) for such purposes.

Conclusion and future prospects

The concept of synaphic targeting with a "magic bullet" to treat cancer has been around for 100 years but has not met the high expectations placed on it. There appear to be several reasons for the modest success of the approach. One is the early focus on targeting the tumor cells, which has been largely stymied by poor penetration of the tumor cell–binding probes into extravascular tumor tissue. Targeting molecular markers that are specific for tumor vasculature does not suffer from this limitation. The realization that the vasculature is more accessible to molecular probes has been a significant advance. There are several compounds that target receptors in tumor vasculature and, in some cases, both tumor vasculature and tumor cells. However, poor tumor penetration still limits the activity of these compounds. The recently described tumor-penetrating peptides may solve the problem with access to the extravascular tumor tissue. The second major limitation of synaphic targeting discussed in this review is the limited capacity of the receptors to which the targeting probes bind, especially when most of the receptors are unavailable for binding because of limited penetration of the probes into tumor tissue. The tumor-penetrating peptides can make receptors available in parts of a tumor not accessible to conventional probes. Finally, tumor-homing nanosystems that amplify tumor homing can also improve the delivery of compounds to tumors, providing imaging and therapeutic options that were previously unavailable.

We thank Dr. Eva Engvall, Dr. Kazuki Sugahara, Dr. Tambet Teesalu, and Kevin Lin for contributions to the manuscript.

E. Ruoslahti, S.N. Bhatia, and M.J. Sailor are jointly supported by the National Cancer Institute Bioengineering Research Partnership (grant CA-122427). The original work reported in this review was also supported in part by an Innovator Award from the Department of Defense (grant W81XWH-08-1-0727 to E. Ruoslahti) and by the National Science Foundation (grant DMR-0806859 to M.J. Sailor). E. Ruoslahti, S.N. Bhatia, and M.J. Sailor are members of the Moores Cancer Center (University of California, San Diego [UCSD], La Jolla, CA) and the NanoTUMOR Center (UCSD), which is supported by the National Institutes of Health (grant U54 CA-119335). S.N. Bhatia is a member of the Harvard/Massachusetts Institute of Technology Center for Cancer Nanotechnology Excellence (Cambridge, MA; grant U54 CA-119349). We have been engaged in collaboration that includes tumor biologists, materials scientists, and bioengineers for the past nine years. We have found it to be both effective and stimulating to work toward a shared goal of improving the outcomes for cancer patients.

Submitted: 19 October 2009
Accepted: 19 February 2010

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