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Combinatorial Discovery of Tumor Targeting Peptides Using Phage Display

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Abstract Peptides possess appropriate pharmacokinetic properties to serve as cancer imaging or therapeutic targeting agents. Currently, only a small number of rationally-derived, labeled peptide analogues that target only a limited subset of antigens are available. Thus, finding new cancer targeting peptides is a central goal in the field of molecular targeting. Novel tumor-avid peptides can be efficiently identified via affinity selections using complex random peptide libraries containing millions of peptides that are displayed on bacteriophage. In vitro and in situ affinity selections may be used to identify peptides with high affinity for the target antigen in vitro. Unfortunately, it has been found that peptides selected in vitro or in situ may not effectively target tumors in vivo due to poor peptide stability and other problems. To improve in vivo targeting, methodological combinatorial chemistry innovations allow selections to be conducted in the environment of the whole animal. Thus, new targeting peptides with optimal in vivo properties can be selected in vivo in tumor-bearing animals. In vivo selections have been proven successful in identifying peptides that target the vasculature of specific organs. In addition, in vivo selections have identified peptides that bind specifically to the surface of or are internalized into tumor cells. In the future, direct selection of peptides for cancer imaging may be expedited using genetically engineered bacteriophage libraries that encode peptides with intrinsic radiometal-chelation or fluorescent sequences. J. Cell. Biochem. 90: 509–517, 2003.

Key words: bacteriophage display; peptide; tumor targeting

Development of agents that specifically bind to tumor cells and their metastases is crucial for improved cancer detection and therapy. Although most cancers are treatable prior to metastasis, cancer in beginning stages is often asymptomatic. As such, early recognition of the primary tumor and efficacious targeting of metastases are critical to successful treatment. While labeled antibody and rationally derived peptide analogues, specific for known tumor-associated receptors, show promise as diagnostic cancer imaging or therapeutic agents, they are only a small number of molecules and target only a limited subset of antigens. Thus, finding new cancer targeting vehicles is a central goal in the field of molecular targeting.

As an alternative to rationally developed peptides, recent efforts to identify new targeting molecules and novel target antigens on carcinomas and metastases have focused on the use of combinatorial technologies for the preparation and screening of "chemical libraries" as sources for new anti-cancer agents [Houghten et al., 1991]. Molecular genetic combinatorial technologies, such as bacteriophage (phage) display, in

Abbreviations used: cp, coat protein; EphA2, ephrin-A1 receptor; ErbB2, epidermal growth factor receptor, type 2; FGF-R, fibroblast growth factor 2 receptor; IC_{50}, concentration at which 50% loss of viability occurred; K_d, equilibrium binding constant; KDR/Flk-1, vascular endothelial growth factor receptor; LC_{50}, concentration at which 50% of cells died; MAP, multiple antigen peptide; MMP, matrixmetalloproteinase; α-MSH, α-melanocyte-stimulating hormone; phage, bacteriophage; scid, severe combined immune deficiency; TF, Thomsen–Friedenreich; TRAMP, transgenic adenocarcinoma of murine prostate; uPA, urokinase plasminogen activator; uPAR, uPA receptor; Pab, peptabody; universal one- and three-letter amino acid abbreviations are used.

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which peptides are displayed on the surface of modified bacterial viruses (i.e., phage), have provided a powerful approach to discovery of new tumor-avid molecules. The ability to select from among the many millions of peptides contained in these libraries in vivo in tumor-bearing animals will greatly facilitate the drug discovery process. This exciting approach will identify new targeting peptides with optimal in vivo stability and can also be expected to identify new classes of tumor biomarkers. Phage libraries engineered to encode radiometal chelation or fluorescent peptide sequences will directly impact their use in cancer imaging.

PEPTIDES ARE EFFECTIVE TUMOR CELL TARGETING AGENTS

Peptides can serve as powerful molecular probes that may be used in the diagnosis and treatment of patients with cancer [Heppeler et al., 2000]. Peptides that bind to tumor cell-associated markers are being actively pursued in imaging and therapy studies because they can possess the required pharmacokinetic properties (high affinity, specificity, and tumor uptake) to serve as cancer imaging agents.

Traditionally, peptide-based targeting agents have been identified rationally by exploiting, through laborious structure–activity studies, peptide ligands for receptors or antigens that are known to be over-expressed on carcinoma cells. One of the best examples of these is Octreotide, which is an octomer peptide analogue of the hormone somatostatin that is in clinical use for imaging somatostatin receptor positive tumors including neuroendocrine, lymphoma, thyroid, and breast tumors [Heppeler et al., 2000]. Other peptide analogues currently being employed include bombesin/gastrin releasing peptide for use in the detection and treatment of prostate, breast, and pancreatic cancer [Van de Wiele et al., 2001] and neurotensin analogues [Hillairet De Boisferon et al., 2002] for colon and other cancers. CCMSH (Cys–Cys–Glu–His–D-Phe–Arg–Trp–Cys–Arg–Pro–Val), a melanoma-targeting, cyclized peptide derivative of the natural α-melanocyte-stimulating hormone (α-MSH) pharmacophore, has recently been developed [Chen et al., 2002]. This peptide targets over-expressed α-MSH receptors in melanomas in vivo with high specificity [Chen et al., 2002].

While these regulatory peptides have been useful, they are but a small subset of the possible targeting peptides for cancer-associated markers. To expand the repertoire of targeting peptides, many researchers have turned to combinatorial chemistry techniques as a basic discovery strategy.

COMBINATORIAL CHEMISTRY FOR ISOLATION OF NEW TUMOR-TARGETING MOLECULES

Combinatorial chemistry is an increasingly powerful approach to modern day drug discovery. In the simplest concept, combinatorial chemical libraries are collections of molecules that can be both chemically or biologically (genetically) synthesized and screened for a desired function or affinity [Houghten et al., 1991]. For the purpose of basic cancer research and cancer drug discovery, there are potential advantages in the use of genetically encoded combinatorial libraries. These libraries are generally displayed on the surface of modified phage. Phage display technology was reported nearly 20 years ago by George P. Smith [Smith, 1985], who, 5 years later, constructed the first phage library by fusing random peptide sequences to a coat protein [Scott and Smith, 1990]. Phage display libraries are typically generated by subcloning random oligonucleotides in frame into the N-terminus of minor coat protein (cp) III (cpIII), or major coat protein VIII (cpVIII), genes of a filamentous phage, such as fd [Scott and Smith, 1990] so that a fusion coat protein is displayed on the solvent-exposed end of the protein (~5 copies/virion on cpIII, >1,000 copies/virion on cpVIII). The vital advantage of surface exposure is that it allows easy surveillance, via affinity selection, of vast numbers of phage clones whose displayed peptides bind specifically to any given molecular target—antibody, receptor, or even tumor (Fig. 1).

To accomplish affinity selection, the library is passed over target molecules, binding clones are captured, and non-binding clones are washed away. The captured phage are infective and can be propagated and cloned at will by infecting fresh bacterial host cells. The primary structure of the foreign peptide is determined easily by sequencing the peptide-coding sequence in the viral DNA. Affinity selection avoids the need to laboriously screen clones one-by-one. Consequently, the number of structures that

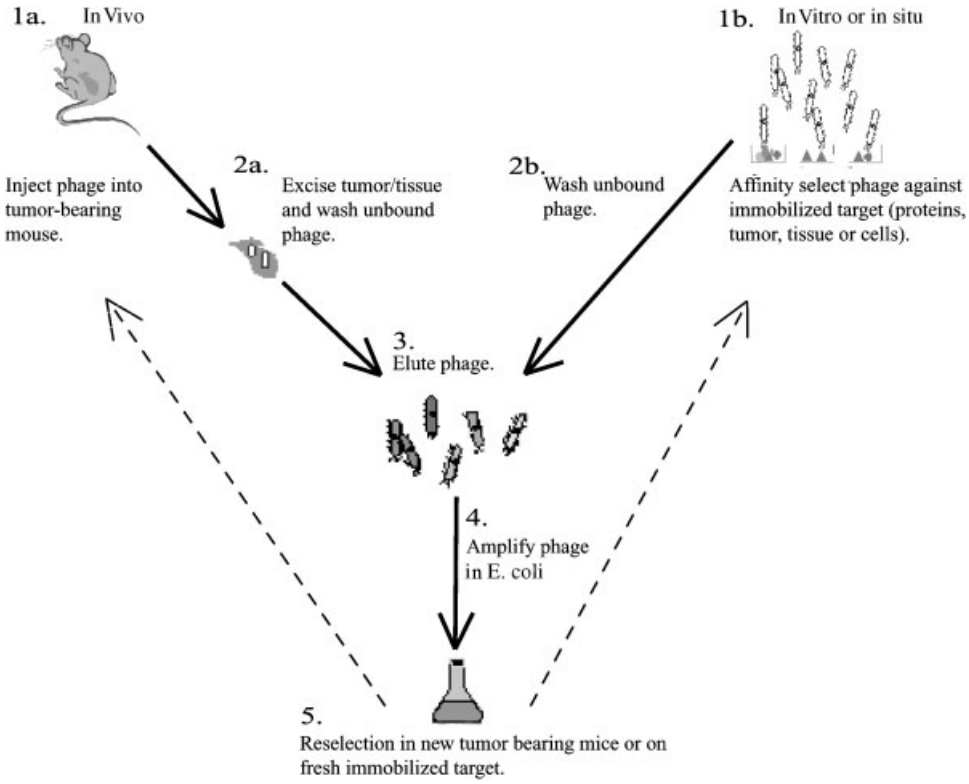

Fig. 1. A schematic diagram of in vitro and in vivo phage display selection of tumor targeting phage clones and resulting deduced peptide sequences. In our laboratory's in vivo selection strategy, random 15 amino acid peptide phage libraries in fUSE5 (gift of George Smith) are initially passaged (not shown) through non-tumor bearing mice (pre-clearance). **1a**: The pre-cleared amplified library (10^{12} transducing units) is selected for 1 h in tumor-bearing mice and tissue is excised. **1b**: Phage are added to immobilized target (proteins, cultured cells, tumor biopsies, tissue sections) and allowed to bind. **2a**: Tumors or tissues are excised from mouse. **2b**: Unbound phage are washed from the plates. **3**: To elute phage from excised tumors and tissues, various elution conditions, including several detergents, are used to retrieve the greatest number of high affinity, specifically-bound phage. Phage bound to immobilized targets (proteins, cultured cells, tumor biopsies) are eluted with low pH buffer. **4**: Phage are amplified in fresh *E. coli* bacteria. **5**: Phage are reselected in new tumor bearing mice or on fresh immobilized target.

can be surveyed is enormous—commonplace libraries contain billions of clones and much larger libraries could be surveyed, if available.

Since 1990, over a thousand reports have been published using this technique to isolate ligands that bind a myriad of targets including antibodies, enzymes, receptors, carbohydrates, and nucleic acid (reviewed in Zwick et al., 1998). Considerably fewer studies have focused on using phage display to isolate tumor-targeting molecules. However, phage display has been successfully employed to obtain peptides that target tumor vasculature [Arap et al., 1998]; tumor cells [Zhang et al., 2001]; tumor cell surface receptors [Houimel et al., 2001; Karasseva et al., 2002]; and protein or carbohydrate tumor-associated antigens [Peletskaya et al., 1997; Landon et al., 2003].

IN VITRO/IN SITU PHAGE DISPLAY FOR NEW TUMOR-TARGETING PEPTIDES

The majority of tumor-targeting peptides have been identified using in vitro affinity selection procedures with a purified tumor-associated antigen (Table I). In vitro selection was employed to identify peptides that bound to the urokinase plasminogen activator (uPA) receptor (uPAR) [Goodson et al., 1994]. uPAR binds uPA, a serine protease produced at high levels in stormal fibroblast-like cells in melanoma, colon, breast, and prostate cancer. The uPA/uPAR interaction is thought be important in early tumor development and in cell adhesion and invasion leading to metastasis. One of the peptides isolated by Goodson et al. [1994], AEPMPHSLNFSQYLWYT, competed for binding of both a radiolabeled uPA fragment

TABLE I. Peptides Selected by In Vitro and In Vivo Phage Display Technology

Target	Sequence	Affinity	References
	In vitro selected peptides		
Protein/receptor targets			
uPAR	LWXXAr (Ar = Y, W, F, H) XFXXYLW	$IC_{50} = 0.01–10$ μM	[3]
ErbB-2	KCCYSL	$K_d = 30$ μM	[4]
	Pab-MARSGL	$K_d = 10$ nM	[5]
	Pab-MARAKE	$K_d = 9$ nM	[5]
	Pab-MSRTMS	$K_d = 16$ nM	[5]
MMP9	CRRHWGFEFC	$IC_{50} = 10$ μM	[6]
MMP2	CTTHWGFTLC	$IC_{50} = 10$ μM	[6]
Kinase domain receptors			
EphA2	SWLAYPGAVSYR, YSAYPDSVPMMS	$K_d = 678$ nM	[7]
		$K_d = 186$ nM	[7]
KDR/Flk-1	HTMYYHHYQHHL	ND[1]	[8]
Cultured cell surface targets			
Human neuroblastoma	VPWMEPAYQRFL	$IC_{50} = 50–80$ μM	[9]
	HLQIQPWYPQIS	$IC_{50} = 0.2–0.3$ μM	[9]
Human embryonic retinoblast (FGF-R)	MQLPLAT	ND[1,2]	[10]
Human malignant glioma	MCPKHPLGC (phage displayed)	ND[1]	[11]
Human prostate carcinoma	DPRATPGS	ND[1]	[12]
Carbohydrate targets			
Thomsen–Friedenreich antigen	HGRFILPWWYAFSPS	$K_d = 1.2$ μM	[13]
	YYAWHWYAWSPKSV	$K_d = 70$ nM	[13]
Lipopolysaccharide	RVVKESR	ND[1]	[15]
	YSALEEG	ND[1]	[15]
	MMGVGTS	ND[1]	[15]
	In vivo selected peptides		
Tumor- or organ-targeting peptides			
Prostate carcinoma	IAGLATPGWSHWLAL GTRQGHTMRLGVSDG	ND[1]	(Fig. 2)
Brain endothelium	CLSSRLDAC	ND[1]	[16]
Endothelium/α_v integrin	ACDCRGDCFCG ("RGD" motif)	ND[1]	[17]
		$LC_{50} = 10$ μM	[18]
Endothelium expressing aminopeptidase N/CD13	CNGRC ("NGR" motif)	ND[1]	[17]
		$LC_{50} = 34–481$ μM	[18]
Breast endothelium/aminopeptidase P	CPGPEGAGC	ND[1]	[19]
Prostate endothelium	SMSIARL	ND[1]	[20]
Lung endothelium (membrane dipeptidase)	CGFECVRQCPERC	ND[1]	[21]
Skin endothelium	CVALCREACGEGC	ND[1]	[21]
Human vascular-targeting tri-peptide motifs			
Prostate endothelium/interleukin-11 protein	AGG	ND[1]	[22]
Skin endothelium	HGG	ND[1]	[22]

Abbreviations used in table: EphA2, ephrin-A1 receptor; ErbB2, epidermal growth factor receptor, type 2; FGF-R, fibroblast growth factor 2 receptor; IC_{50}, concentration at which 50% loss of viability occurred; K_d, equilibrium binding constant; KDR/Flk-1, vascular endothelial growth factor receptor; LC_{50}, concentration at which 50% of cells died; MMP, matrixmetalloproteinase; Pab, pentabody; uPAR, urokinase plasminogen activator receptor; universal one-letter amino acid codes are used. [1]Not determined. [2]Affinity of phage for SKOV cells, $K_d = 1.51 \times 10^{11}$ plaque forming U/ml. References: [3][Goodson et al., 1994], [4][Karasseva et al., 2002], [5][Houimel et al., 2001], [6][Koivunen et al., 1999], [7][Koolpe et al., 2002], [8][Hetian et al., 2002], [9][Zhang et al., 2001], [10][Maruta et al., 2002], [11][Spear et al., 2001], [12][Romanov et al., 2001], [13][Peletskaya et al., 1997], [14][Landon et al., 2003], [15][Noda et al., 2001], [16][Pasqualini and Ruoslahti, 1996], [17][Arap et al., 1998], [18][Ellerby et al., 1999], [19][Essler and Ruoslahti, 2002], [20][Arap et al., 2002a], [21][Rajotte et al., 1998], [22][Arap et al., 2002b].

($IC_{50} = 0.01$ μM) and for phage displaying either the cognate or other uPAR-binding peptides (Table I). However, uPAR is also expressed on non-carcinoma cells, which underscores the problems faced in designing specific anticancer therapies based on receptor–ligand systems not restricted to carcinoma cells.

The epidermal growth factor receptor, type 2 (ErbB-2) tyrosine kinase is a carcinoma-associated receptor that shows increasing promise as a target for cancer diagnosis and therapy. The ErbB-2 protein is encoded by the erbB-2 proto-oncogene (HER-2, neu). Over-expression of ErbB-2 is associated with increased tumor growth and metastases in many human cancers, most notably breast and prostate [Qiu et al., 1998]. ErbB-2-binding peptides have been selected by using phage display (Table I). One group reported a peptide, KCCYSL, which bound to purified ErbB-2 with a K_d of 30 μM, and to breast and prostate cancer cell lines, but not to normal cells [Karasseva et al., 2002]. Houimel et al., isolated ErbB-2-binding linear peptide monomers (MARSGL, MARAKE, MSRTMS) (Table I), from which humanized pentameric "peptabody" (Pab) molecules (linear peptide sequences fused

to an antibody-like tail that allows pentamerization) were derived. The Pab bound to ErbB-2 with $K_d \cong 10$ nM and was able to inhibit growth and proliferation (40% inhibition) of ErbB-2-expressing cultured cells [Houimel et al., 2001].

Cell surface carbohydrate antigens, which are among the many molecules thought to be involved in cancer and metastasis, are receiving much attention because alterations in their composition can be associated with malignant transformation and provide a unique molecular signature that may be exploited for cancer detection or treatment. Noda et al. [2001] used phage display to identify peptides specific for carbohydrates, including lipopolysaccharide. Competition experiments demonstrated that phage displaying three peptides (RVVKESR, YSALEEG, MMGVGTS) bound to different sites on the core oligosaccharide structure of the *Salmonella* lipopolysaccharide molecule [Noda et al., 2001].

We have employed in vitro phage display to affinity select for peptides that bound to the Thomsen–Friedenreich (TF) tumor-associated antigen [Peletskaya et al., 1997; Landon et al., 2003]. TF antigen was found to consist of a terminal galactose β1-3 *N*-acetylgalactosamine carbohydrate moiety that was exposed on the outer surface membranes of ~90% of primary carcinomas, lymphomas, and their metastases, and correlated with increased metastatic potential [Springer et al., 1975]. One of the in vitro selected first generation peptides (HGRFILPWWYAFSPS) (Table I, Fig. 2), bound specifically to carcinoma cells and inhibited the adhesion of breast and prostate cancer cells to human bone marrow endothelial cells [Peletskaya et al., 1997], which suggested that TF antigen-mediated interactions might be important in tumor cell adhesion to bone marrow vasculature [Glinsky et al., 2001]. The carbohydrate-binding peptide monomer had millimolar affinity for TF antigen (Fig. 2), which limited its use [Peletskaya et al., 1997]. Subsequently, two different strategies have been taken to improve the peptide monomer's affinity and solubility. First, a novel directed combinatorial affinity maturation procedure was applied to the TF antigen-avid core consensus sequence (WYAW/FSP) [Landon et al., 2003]. The new generation of peptides, which had improved affinity (~10 fold) (Fig. 2) and water solubility, will be explored as potential tumor imaging and targeting agents. In the second strategy to improve peptide function, multiple antigen peptide (MAP) constructs of the maturated peptides were synthesized and had picomolar to femtomolar affinity for TF (Fig. 2).

The use of intact cancer cells or tissues as a source of material for phage library affinity selection was recently reported. One group employed phage display to select for neuroblastoma-binding peptides in situ [Zhang et al., 2001]. A random 15 amino acid phage library was selected against the human neuroblastoma

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Peptide	Affinity
First generation	1.2 μM
Maturated	59 nM
MAP	798 fM

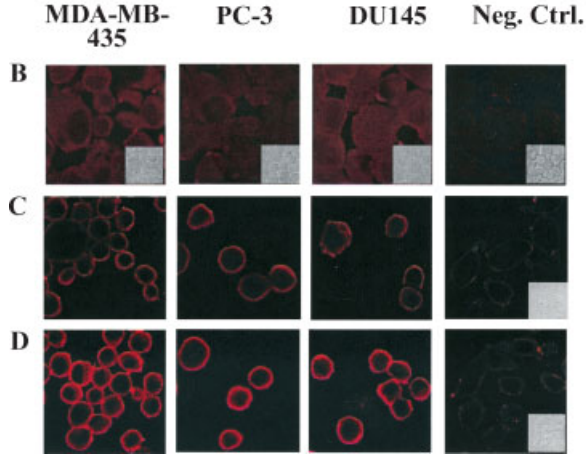

Fig. 2. Combinatorial maturation of in vitro-selected anti-TF antigen peptides. Random 15 amino acid peptide libraries in fUSE5 phage (gift of George Smith) were affinity-selected against immobilized TF antigen displayed on bovine serum albumin. Peptides (first generation), encoded by the selected phage, were synthesized and an anti-TF antigen consensus sequence was identified. Functionally maturated peptides (maturated) were selected by using directed combinatorial affinity maturation. Peptides, in which the anti-TF antigen consensus sequence was held constant and was surrounded by N- and C-terminal domains of random amino acid sequence, were displayed on fUSE5 phage and selected against TF antigen displayed on human serum albumin. One maturated sequence was used to design a MAP construct. **A**: Affinity of binding of all peptide molecules was determined by using fluorescence quenching titrations. **B**–**D**: Binding of the first generation peptide (B), the maturated peptide sequence (C), and the MAP construct of the same maturated sequence (D) to cultured human carcinoma cells. Fixed, permeabilized cultured human breast (MDA-MB-435) and prostate (PC3, DU145) carcinoma cells were immobilized on a glass microscope slide and incubated with 20 μM solutions of biotinylated peptides or secondary ligand only (Neg. Ctrl.). Binding was detected with NeutrAvidin-Texas Red and laser scanning confocal microscopy. At the concentration used (20 μM), no binding of the first generation peptide to any cell line was visible.

cell line, WAC 2 (Table I). One peptide, VPWMEPAYQRFL, bound both breast and neuroblastoma cancer cell lines ($IC_{50} = 50-80\ \mu M$), but not normal cells. However, the majority of peptides bound normal cell lines in addition to cancer cell lines, which highlights the practical problems in identifying specific and functionally important peptides using affinity selection. Petrenko and co-workers [Romanov et al., 2001] screened a novel phage landscape library against the human prostate carcinoma cell line, LNCaP, in an effort to discover biologically active ligands. Landscape libraries are phage peptide libraries, in which different surface structures ("landscapes") are generated by fusing random octapeptides to the N-terminus of all 4,000 copies of the major coat protein, cpVII. Such a "landscape library" may include clones that exhibit structural/functional properties that can emerge only in the context of the phage surface architecture [Romanov et al., 2001]. From the landscape library, numerous peptides were selected. One peptide, pg35 (DPRATPGS), was found to block the spreading of LNCaP-parental and -derived cells (Table I). The peptide activated matrix metaloproteinases (MMP)-2 and -9 in the LNCaP-derived cells, suggesting that the peptide ligand plays a role in tumorigenicity and metastasis [Romanov et al., 2001].

IN VIVO PHAGE DISPLAY

An underlying problem with in vitro and in situ selections of phage peptide libraries is that, while the peptides often possess high affinity for the target antigen in vitro, they may not effectively target the tumor in vivo. This may be due to low antigen expression in the tumor or problems with peptide stability and specificity, which one cannot easily select against. To solve this dilemma, innovative in vivo combinatorial chemistry methods are being developed. The in vivo application of combinatorial libraries has the advantage over in vitro selection strategies in that one can "select," in the environment of the whole animal, peptides that bind selectively and stably to a given tumor. Rouslahti and Pasqualini pioneered this approach using live mice to select for organ-targeting peptides [Arap et al., 1998]. Several peptides, particularly those that bind to tumor vasculature were discovered (reviewed in Trepel et al., 2002). In particular, their group reported a plethora of peptides with RGD or NGR motifs (Table I), which targeted molecules on organs and tumor vasculature [Pasqualini and Ruoslahti, 1996]. When coupled to doxorubicin, the RGD integrin-targeting peptide (ACDCRGDCFCG) increased the efficacy of doxorubicin in inhibiting tumor growth while reducing non-specific toxicity to the animal [Arap et al., 1998]. When linked to a pro-apoptotic peptide (KLAKLAKKLAKLAK), both the RGD peptide (ACDCRGDCFCG) and the NGR peptide (CNGRC) were more effective in reducing proliferation of KS1767 cells, (derived from Kaposi's sarcoma) than the pro-apoptotic peptide alone (RGD, $LC_{50} = 10\ \mu M$; NGR, $LC_{50} = 42\ \mu M$, pro-apoptotic peptide, $LC_{50} = 387\ \mu M$) (Table I). More recent studies have focused on analyzing peptides that target to specific organ vasculature [Essler and Ruoslahti, 2002]. A peptide, SMSIARL (Table I), isolated from phage peptide libraries selected in transgenic adenocarcinoma of murine prostate (TRAMP) mice, specifically targeted prostate tumor vasculature, and when injected into mice effectively reduced tumor size [Arap et al., 2002a]. Their group also identified a cyclic peptide, CPGPEGAGC that bound aminopeptidase P, which was selected from mouse mammary tissue in vivo [Essler and Ruoslahti, 2002]. Taken together, these studies suggested that individual organs may have unique vasculature signatures [Trepel et al., 2002].

Based on their work, a multi-national consortium of researchers is using phage peptide display to map in vivo tissue-specific vascular addresses in humans [Arap et al., 2002b]. A Cys–X7–Cys disulfide-constrained, random peptide phage library was injected into a brain dead human and biopsy samples were obtained from the major organs and tissues. Greater than 40,000 peptide motifs were identified and analyses indicated that the biodistribution of the circulating phage was nonrandom. Many of the sequences exhibited structural and distribution similarities to known ligands for proteins that are differentially expressed on selected tissues, such as the bone marrow, fat, prostate tissue, and skin (Table I) [Arap et al., 2002a]. In vivo selected peptides, such as these, may likely target and bind to organ- and tumor-specific vascular addresses to specifically deliver cytotoxic drugs or imaging reagents to the vascular address of the tumor.

While the vasculature-targeting peptides may yield important insights into vascular biology, angiogenesis, and tumor growth, there

remains a need to develop new peptides that target antigens restricted to carcinoma cells and their metastases. It can be argued that if one desires to develop new cancer-specific imaging and/or therapeutic biomolecules, it may be meaningful to select peptides in vivo that target the cancer cell, rather than the surrounding vasculature. The in vivo selection schemes reported by Pasqualini and co-workers [Ellerby et al., 1999] may have preferentially selected for vasculature homing peptides with common motifs present in the libraries because of short incubation times (15 min) in animals. In addition, clone-by-clone propagation of a miniscule portion of the entire phage library was applied. This may result in only a small proportion of the infectious units in the target tissue being amplified for the next round of selection. This is likely to favor very short motifs, such as RGD, present in many independent clones in the initial random peptide library, rather than longer, higher affinity, and more specific motifs, which are very rare in the initial library.

We have developed in vivo selection schemes that effectively yield phage that display uncommon peptides, which target carcinomas, penetrate the tumor, and can internalize into the carcinoma cells (Fig. 1). To accomplish this, phage libraries were first pre-cleared in non-tumor-bearing mice to reduce vasculature- or other organ-targeting phage. Pre-cleared libraries were injected into prostate tumor-bearing severe combined immune deficiency (scid) mice and were allowed to circulate for times sufficient to eliminate unstable or circulating phage (1 h). Phage were eluted from tumors using a series of detergent extractions. Furthermore, the entire phage population captured in the target tumor was amplified. Results of the selections revealed that those phage clones that were eluted from the prostate tissue with the highest concentration of detergent had the highest affinity and specificity for the original cancer cell line used in tumor production (G1, IAGLATPGWSHWLAL) and were in some cases, internalized into the cancer cells (D4, GTRQGHTMRLGVSDG) (Fig. 3). Thus, in vivo phage library selection strategies can be augmented to obtain peptides that bind the cancer cell and may also be selected on their ability to internalize into the cell. The antigens "targeted" by this approach may be previously identified tumor antigens, but more likely will represent new classes of tumor biomarkers for future exploration.

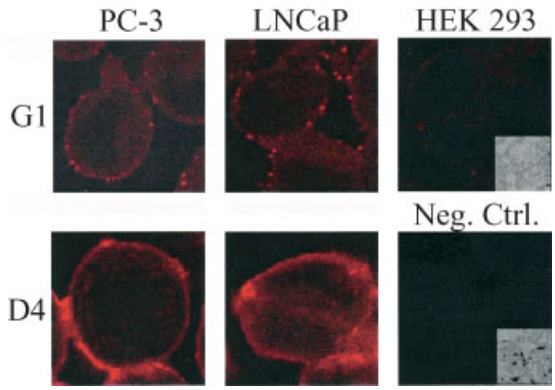

Fig. 3. In vivo-selected peptides, which were encoded by the phage clones with the highest titers and thus have the theoretically highest affinity for the tumor, were examined for their ability to bind tumor and control cell lines using immunocytochemistry. Fixed, permeabilized cultured human prostate carcinoma cells (PC-3, LNCaP) and cultured human embryonic kidney cells (HEK, negative control cell line) were immobilized on a glass microscope slide and incubated with 100 μM solutions of biotinylated peptides (G1, IAGLATPGWSHWLAL; D4, GTRQGHTMRLGVSDG) or secondary ligand only (Neg. Ctrl.). Binding was detected with NeutrAvidin-Texas Red and laser scanning confocal microscopy.

APPLICATION OF PEPTIDES TO TUMOR IMAGING AND THERAPY

Peptides selected by using combinatorial phage display can be synthesized and subsequently radiolabeled using well-developed chelation strategies. These are then used as targeting molecules to direct therapeutic and/or imaging agents to primary tumors and their metastases. However, post-synthetic labeling may destroy the binding and targeting functions of the small peptide. To address this problem, new intrinsic labeling strategies are being developed, which hold great promise for radioactive and fluorescent labeling of targeting peptides. Targeting molecules have been labeled with intrinsic "reporter" molecules for positron emission tomography and fluorescent optical imaging for noninvasive molecular imaging (reviewed in Blasberg, 2002). Alternatively, phage display libraries can be engineered to express peptides with genetically encoded radiometal chelation sequences, such as CysCysGlyCys [Karasseva et al., 1999] or poly-His [Waibel et al., 1999]. Using these approaches, peptides can be selected for high affinity and specific binding in the presence of the chelation group, which obviates the problem of destruction of peptide function or tumor-targeting ability by the chelate or functional group.

Combinatorial phage display technology provides a powerful molecular genetic method for realizing the central goal of molecular targeting the discovery and application of new tumor-avid molecules. This approach offers the unique in vivo opportunity to select, from among many millions of peptides, new targeting peptides with optimal in vivo affinity, specificity, and chemical stability. Furthermore, selection of targeting peptides with intrinsic radiometal chelation or fluorescent reporters will revolutionize the use of combinatorially derived peptides in cancer imaging.

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