REVIEW

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Molecular addresses of tumors: selection by *in vivo* phage display

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ABSTRACT

In vivo phage display has been used extensively to screen for novel targets of tumor therapy. Phage display peptide libraries can express random peptides or protein fragments and the aim of phage display is to identify peptide molecules that bind stably to a given target. Angiogenesis is essential to tumor development. Both blood and lymphatic vessels of tumors are different from those of normal tissues. Phage display has been used to analyze the structure and molecular diversity of tumor vasculature and to select tumor-specific antigens which have revealed stage- and type-specific markers of tumor blood vessels. Furthermore, peptides identified by *in vivo* phage display also work as vehicles to transport cargo therapeutic reagents to tumors. These peptides and their corresponding cellular proteins and ligands may provide molecular tools to selectively target the addresses of tumors and their pathological blood vessels and might increase the efficacy of therapy while decreasing side effects.

Key words: phage display, tumor therapy, angiogenesis, tumor antigen.

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INTRODUCTION

Different organs express a unique set of functional molecules that determine the endothelial heterogeneity of the organ and distinguish it from other organs. Thereby, a zip code of molecular addresses is provided for selective homing of cells, i.e. particular leukocytes in the blood stream. During disease, changes in this zip code system are due to the organ-specific patterns of inflammatory or other pathological responses.

Angiogenesis is essential to tumor development and the tumor vasculature expresses unique markers that distinguish it from normal vasculature. Furthermore, stage-specific molecular changes might accompany carcinogenic progression and, finally, metastasis. Therefore, strategies based on inhibition of angiogenesis are considered of great promise. The structural and molecular diversity of tumor-associated vasculature provides a basis for the development of targeted diagnostics and therapeutics [58]. Screening of phage display peptide libraries *in vivo* is an alternative and complimentary tool to rapidly identify specific markers of tumor vasculature. A phage display peptide library can express random peptides or protein fragments homing to the tumor vasculature and directly binding to the molecules expressed by tumor vessels [3, 43].

In vivo peptide phage display screening has the advantage of identifying organ-specific, functionally active proteins of tumor vasculature. The selected peptides might be used as selective vehicles transporting chemotherapeutic agents to the tumor vasculature, thus improving drug specificity and efficacy and reducing toxicity. In addition, some peptides selected by phage display are bioactive by themselves and block the function of their ligand proteins [48]. Consequently, peptides selected by phage display have been used to image, identify, control, and treat a large range of tumors, including thyroid tumors, rat glioblastoma, prostate cancer, malignant melanoma, and leukemia [9, 10, 52, 53, 57].

PHAGE DISPLAY IN THE ANALYSIS OF VASCULAR ADDRESSES

In vivo phage display has been repeatedly used to analyze the structure and molecular diversity of tumor

vasculature [44]. The process of *in vivo* phage display includes several steps: injection of unselected phage display libraries, rescue of recombinant phages from the target tissues, and phage amplification by infection of *Escherichia coli* cultures. This process is repeated several times to obtain phages specifically binding to the target tissue. Furthermore, sequencing, immunohistology, Western blot analysis, or flow cytometric analysis are necessary to validate phages and their corresponding targets [23, 38, 52].

Tumor cell homing depends on an adhesive interaction between tumor cells and organ-specific endothelial markers [50]. Phage screening and monoclonal antibody studies have suggested that every tissue may express highly specific markers in its vasculature [2, 16, 24, 50]. Neoangiogenesis plays an important role in tumor progression, and endothelial cells within the tumor express a stimulated phenotype which is associated with the variable expression of cell surface molecules [41]. Molecules capable of selectively targeting markers of angiogenesis may offer opportunities for the *in vivo* mapping of aggressive tumors and for the delivery of toxic agents to the tumor vasculature [55]. Until now, several peptides specifically binding to such markers have been investigated (Table 1). Phage display technology was used to identify stage-specific tumor-vessel zip codes, and it has been reported that characteristics of the angiogenic vasculature distinguish premalignant and malignant stages of skin tumorigenesis [22, 48]. Thus the tumor vasculature expresses stage-specific molecules during carcinogenic progression and the identification of this set of unique molecular markers associated with different stages of progression to malignancy will be very important in tumor therapy and diagnosis. Peptides homing to the tumor vessels during premalignancy are potential reagents for early tumor diagnosis and localization and early treatment before metastasis. Such peptides may also block the functional protein, and could thus be considered anti-tumor agents.

Lymphatic vessels are abundant in the periphery of tumors and many tumors also have lymphatics in the tumor mass. Several reports suggest that tumor lymphatics carry specific markers and these may be possible targets of tumor therapy [25, 33]. Recent experimental and clinical data strongly suggest that lymphatic tissues determine tumor metastasis notably by expression of lymphoangiogenic growth factors [36, 46, 51, 54]. Laakkonen et al. [32] investigated the antitumor activity of LyP-1 selected from a phage display peptide library and revealed that this peptide can bind to tumor and endothelial cells of tumor lymphatics in certain tumors and showed antitumor activities. Given that tumor lymphatics have not been shown to be important for tumor growth [21], the anti-tumor effect of LyP-1 may more likely affect tumor cells within and close to the lymphatics, the cells most likely to spread through the lymphatic system [29].

Table 1. Peptides selectively binding to vascular endothelial addresses

Target marker	Sequence of peptide selected by phage display	Peptide description
VEGF receptor-2 (Flt-1 or KDR)	ASSSYPLIHWRPWAR [5]	suppresses the migration of VEGF-stimulated human umbilical cord vein endothelial cells
	PVVLFPLH p [4]	suppresses the migration of VEGF-stimulated human umbilical cord vein endothelial cells
	HTMYYHHYQHHL [20]	disrupts the interaction between VEGF and the KDR receptor and causes inhibition of angiogenesis and tumor growth
	WHSDMEWWYLLG [1]	specifically binds to VEGF receptor Flt-1
	SP5.2 [17]	selectively binds Flt-1 and inhibits a broad range of VEGF-mediated events
	ATWLPPR [7]	totally abolished VEGF-induced angiogenesis, blocks VEGF-KDR interaction
	GGNECDAIRMWEWECFERL [42]	selectively binds to KDR and Flt-1
	A2, A7, B11, G3, and H1 [8]	recognizes VEGF receptor-2
	DHFR-F56/F90 [35]	binds to VEGF receptor-2, induces tumor necrosis and inhibits tumor growth *in vivo*
VCAM-1	VHSPNKK [27]	high affinity to endothelial cells expressing VCAM-1, blocks leukocyte-endothelial interactions
EphA2	SWLAYPGAVSYR [31]	binds selectively to EphA2 with high affinity
	YSAYPDSVPMMS [31]	binds selectively to EphA2 with high affinity
Gelatinase	CTTHWGFTLC [29]	inhibits the migration of human endothelial cells and tumor cells
Aminopeptidase N	NGR [45]	binds to aminopeptidase N
Undefined markers	PRPGAPLAGSWPGTS [6, 41]	
	KPSGLTY [37]	binds to the luminal surface of polarized endothelium

VCAM – vascular adhesion molecule, VEGF – vascular endothelial cell growth factor.

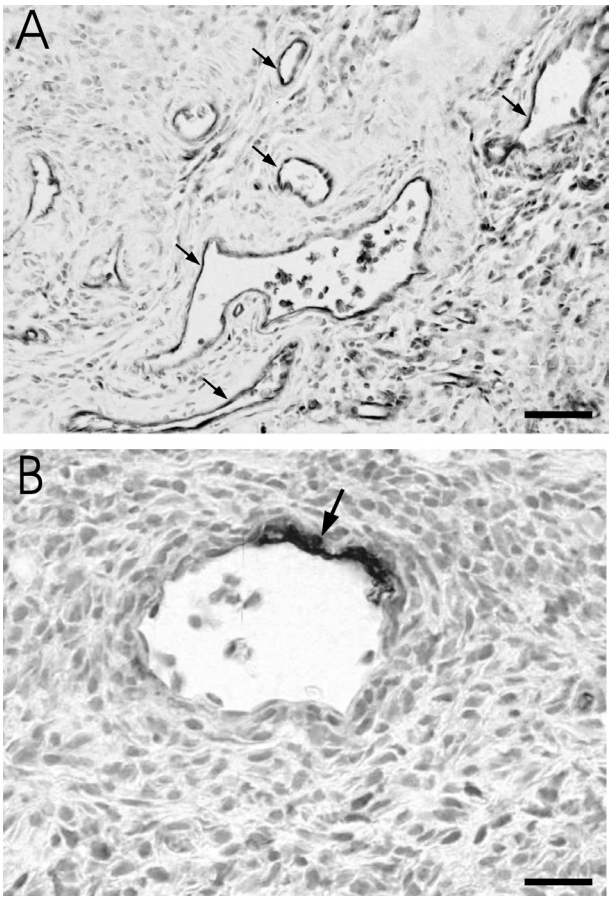

Fig. 1. Immunolocalization of a recombinant M13 phage in rat C6 glioblastoma. **A** – recombinant phages of this clone only bind to tumor vasculature but not to normal brain vessels, but not all tumor blood vessels were stained. **B** – M13 phages showed asymmetric staining in individual vessels revealing the variability of tumor vasculature (bars: A=200 μm, B=50 μm).

IDENTIFICATION OF TUMOR ANTIGEN

Proteins or peptides possessing appropriate pharmacokinetic properties are good candidates to serve as anti-tumor agents; however, the peptides selected might be not effective in targeting tumors *in vivo* due to poor peptide stability and other problems. On the other hand, murine cell-specific monoclonal antibodies (mAbs) are typically discovered by screening numerous hybridomas following the immunization of mice with a target human cell line or antigen [19, 28]. However, as many widely expressed human antigens are foreign antigens for the murine immune system, it is hard to discover most of the tissue- or disease-specific human antigens [56]. Thus, finding cancer-targeting peptides *in vivo* is a central goal of tumor therapy. Now, a novel method to identify peptides efficiently is affinity selection using the phage display technique. Bacteriophages display random peptide libraries which contain millions of peptides. The advantage of phage display is to allow tumor-targeting peptide selection within the environment of the whole tumor-bearing animal [34]. This technology has been used successfully to purify human mAbs against viruses, self antigens, and tumor antigens [11, 12, 39, 40]. Our library has used *in vivo* screening of phage libraries to identify peptides binding to the endothelium of experimental rat brain tumors, the C6 glioblastoma (Fig. 1) [52].

CYTOTOXIC CARGO

Peptides homing to a target site are attractive as carriers of therapeutic and diagnostic agents. Drug delivery by a homing peptide can concentrate the drug at the targeted tissue, increasing efficacy and decreasing side effects to other sites. Ligand-to-cell-membrane adhesion-receptor fusing to the NH2 terminus of tumor necrosis factor (TNF) is able to target TNF to tumors, which has been shown to be more effective and less toxic to normal tissue than TNF alone [14]. Based on a modification of the phage display technology, De Berardinis et al. [15] developed an innovative concept for a safe and inexpensive vaccine to HIV. Their data showed that this system could trigger specific T-cell response *in vitro* and *in vivo*.

Peptides which are capable of being internalized by target cells and of delivering cargo, such as fluorescein, rhodamine, or biotin, into the cell nucleus have been identified [22, 26, 47]. Furthermore, phage display was used to identify homing peptides for blood vessels *in vivo*. Some of these peptides were composed of several basic amino acids which play important roles in the interaction between proteins and targets. The peptides home neither to vessels in normal skin nor other normal organs. In consequence, proteins detected by phage display are proposed as drug carriers for site- or tissue-specific drug delivery.

CONCLUSION

In vivo phage display is a suitable technology to identify unique sets of homing proteins to specific vascular addresses. This technology is extensively applied to image, identify, control, and treat a large range of tumors. Several groups have described specific proteins of the tumor vasculature of different type and stage of malignancy. Some of these proteins are potential candidates for therapeutic applications [13, 18, 30, 49].

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