Vascular zip codes in angiogenesis and metastasis

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Abstract

In vivo **screening of phage-displayed peptide libraries has revealed extensive molecular differences in the blood vessels of individual normal tissues. Pathological lesions also put their signature on the vasculature; in tumours, both blood and lymphatic vessels differ from normal vessels. The changes that characterize tumour blood vessels include selective expression of certain integrins. Peptides isolated by *in vivo* phage display for homing to tumours have been shown to be useful in directing therapeutic agents to experimental tumours. The targeting can enhance the efficacy of the therapy while reducing side effects. Phage screening has also revealed lung-specific vascular markers that promote tumour metastasis to the lungs by mediating specific adherence of tumour cells to the lung vasculature. These phage-screening studies have revealed a previously unsuspected degree of vascular specialization and provide potentially useful guidance devices for targeted therapies.**

Introduction

Vascular zip codes exist in lymphoid tissues, where the specialized endothelial cells of high endothelium express adhesion molecules that direct lymphocyte homing. It is less well known that many, perhaps all, non-lymphoid tissues put a tissue-specific 'signature' on their vasculature [1]. Moreover, endothelial up-regulation of leucocyte adhesion molecules at inflammatory sites and distinct features of the vasculature in tumours are examples of pathological tissue processes that cause changes in the vasculature [1,2].

Tumours stimulate angiogenesis to secure a blood supply for the growing tumour [3,4]. The activated endothelial cells and pericytes in this neovasculature express molecules that are not expressed or are expressed at much lower levels in normal vessels. Moreover, the endothelial cells of tumour lymphatics also express tumour-specific markers [1].

Tissue-selective tumour metastasis can be facilitated by the adhesion of tumour cells to tissue-specific zip code molecules on the surface of endothelial cells [5]. Elucidation of such adhesion mechanisms may provide molecular markers predictive of metastasis and the means of suppressing metastasis. Markers that are specific for tumour vasculature provide new opportunities for targeted delivery of therapies.

Phage display libraries expressing random peptides or protein fragments have been particularly useful in analysing vascular heterogeneity. Several years ago, we initiated *in vivo* screening of phage libraries for the purpose of identifying specific markers in the vasculature of normal tissues and tumours [6,7]. Our results reveal extensive heterogeneity in tumour blood vessels and lymphatics and we have also isolated homing peptides for a large number of individual tissues by using this method. Some of the tissue-specific endothelial markers detected with the homing peptides appear to serve as binding sites for metastasizing tumour cells. Herein, I will briefly discuss recent developments in this area.

In vivo phage display in vascular analysis

Our laboratory has used *in vivo* screening of phage libraries that express random peptides or cDNA-encoded protein fragments to identify tissue- and tumour-specific features of the vasculature. In the early studies, we injected unselected libraries into the tail vein of mice, rescued the phage from the target tissues and, by repeating the process several times, were able to isolate phage that specifically homed to the target tissue. The premise was that the phage particles would not leave the vascular space during the short (approx. 5 min) circulation time and that, as a result, only phage binding to the endothelium (and perhaps other cells in the vessel wall) would be isolated. This conjecture has been proven to be

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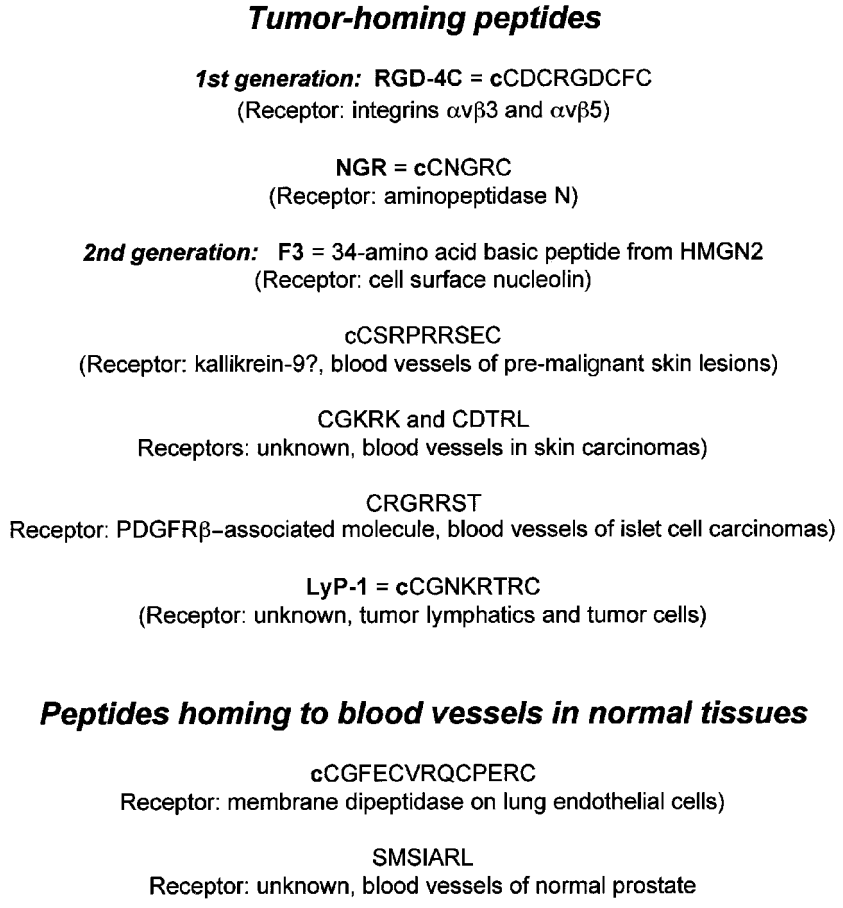
Figure 1 | Vascular homing peptides and their receptors



accurate. The 'first-generation' homing peptides we identified for various normal tissues or tumours bound to the blood vessels at the target [5–7] (Figure 1).

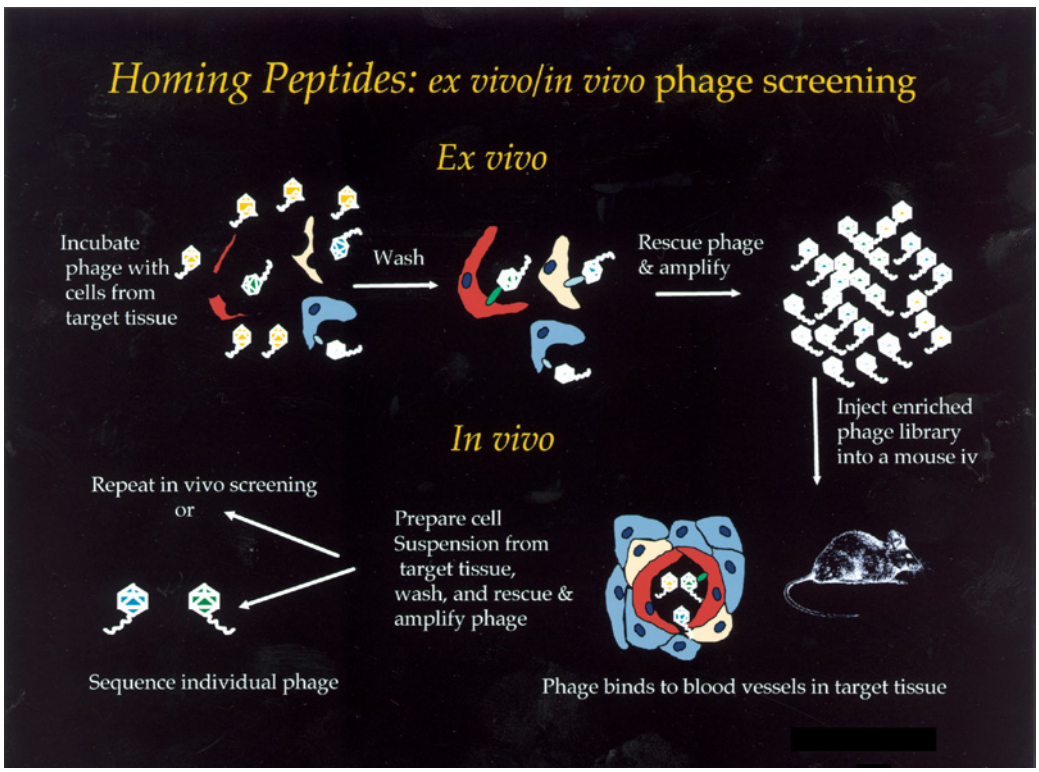
More recently, we have added an *ex vivo* step to the process to enrich the phage library on cell suspension prepared from the target tissue, and use the enriched library for *in vivo* screening [8]. A schematic representation of the *ex vivo/in vivo* phage screening procedure is shown in Figure 2. This *ex vivo/in vivo* procedure appears to favour peptides that are different from the first-generation peptides obtained by screening only *in vivo*. Two features set apart these 'second-generation' homing peptides: they effectively carry a payload to the target tissue as monovalent conjugates and many of them take the payload into the target cell [9–12]. As a result of the cell type-specific internalization, the payload (fluorescein and biotin, so far) becomes concentrated in the target cells. If the concentrating effect can be reproduced in preclinical and clinical settings, targeted therapies with enhanced efficiency may be possible. In this regard, our peptides are similar to the Tat, penetratin and VP22 peptides, which are also efficiently taken up by cells. An important difference is that these peptides are taken up by all cells, whereas our homing peptides are cell type-specific.

Blood vessel specialization in normal tissues

The phage screening studies have uncovered an unexpected extent of tissue-specific molecular heterogeneity in the vasculature of various normal tissues. We have obtained tissue-specific vascular homing peptides for each normal tissue and organ our laboratory has chosen for targeting so far. The list includes both major organs, such as the brain, lungs, heart and kidneys, and minor ones such as the prostate [5,13,14]. With our current technology [8], the selectivity of phage homing to a specific organ can be several hundredfold (L. Zhang, J. A. Hoffman and E. Ruoslahti, unpublished work). These results suggest that every tissue may express specific markers in its vasculature. Less extensive studies with monoclonal antibodies support this conclusion [15,16].

The molecular nature of vascular changes that give rise to the individuality of the vessels in various tissues and pathological lesions is only partially understood. Several proteases have been identified as markers of the vasculature in individual normal tissues. Thus two peptidases (dipeptidyl peptidase IV [17] and membrane dipeptidase [18]), and a chloride channel are selectively expressed in lung vessels

Figure 2 | Schematic representation of the *ex vivo/in vivo* phage screening procedure



[19]. Another peptidase, aminopeptidase P, is a marker for breast gland vasculature [13]. However, the tissue-specific endothelial markers appear to include many other types of molecules as well (L. Zhang, J. A. Hoffman and E. Ruoslahti, unpublished work).

Special features of tumour vessels

Previous studies have shown that tumour vessels express a number of markers that are characteristic of angiogenesis. For example, the expression of receptors for vascular endothelial growth factor is elevated in the endothelial cells of tumour blood vessels [3]. Certain integrins are another group of receptors elevated in angiogenic vessels. The $\alpha v\beta 3$, $\alpha v\beta 5$ and $\alpha 5\beta 1$ integrins are overexpressed in tumour vasculature [20]. Indeed, one of the peptides identified by *in vivo* screening of phage libraries for tumour homing recognizes $\alpha v\beta 3$ and $\alpha v\beta 5$ [7].

Similar to the molecular markers of normal vessels, the angiogenesis markers also include peptidases/proteases. Aminopeptidase N was established as a new marker of angiogenic vessels with our first-generation tumour-homing peptides containing the motif Asn-Gly-Arg [21]. Aminopeptidase N is a membrane protein that is expressed in some epithelial cells and immune cells; however, within the vasculature, it is specific for angiogenesis. Aminopeptidase N has been previously linked to cell migration and tumour invasion, but not to angiogenesis. The form of aminopeptidase N expressed in tumour vasculature may be different from that in normal cells [22]. In a similar vein, an antibody that recognizes a splice variant of fibronectin selectively recognizes the ECM (extracellular matrix) of angiogenic blood vessels [23].

We have designed recently a phage-screening method aimed at yielding peptides that might recognize endothelial precursor cells. In this screen, we first selected phage for binding to bone marrow cells *ex vivo* and then for homing to tumour vasculature [10]. One of the peptides, F3, identified in this manner recognizes a small population of bone marrow cells, angiogenic endothelial cells and certain tumour cells. We have now shown that the molecule the F3 peptide binds to in the target cells is cell-surface-expressed nucleolin [24]. Antibodies prepared against nucleolin also specifically recognize blood vessels in tumours and in a non-malignant angiogenesis model. Thus cell-surface-expressed nucleolin is a new endothelial marker of angiogenic vessels, including those in tumours, where it also recognizes the tumour cells.

An unexpected degree of vascular diversity was revealed in our recent collaborative study with Dr Douglas Hanahan's laboratory [11,12]. Using transgenic mouse tumour models, we showed that pancreatic islet cell tumours and skin tumours expressed different markers in their vasculature. Another novel finding was that some homing peptides distinguished between the vessels of premalignant and malignant lesions (while not recognizing the normal vasculature). This latter

finding indicates that the molecular features of the vasculature reflect the stage of tumorigenesis.

Some of the peptides identified in the transgenic tumour study bound both to the endothelial cells and pericytes in the tumour vasculature. Previous studies have also shown that pericytes in tumour vasculature carry specific markers. The NG2 proteoglycan, also known as melanoma-associated chondroitin sulphate proteoglycan, is one such marker. NG2 is a membrane-spanning cell-surface protein that is expressed in the neovasculature of tumours, regenerating tissues and in foetal vessels [25].

We have reported recently evidence indicating that lymphatic vessels in tumours can also express tumour-specific markers. A nonapeptide, LyP-1, specifically recognizes the lymphatic vessels in some tumours, including xenografts produced with the MDA-MB-435 human breast cancer cell line [9]. LyP-1 does not recognize lymphatics in normal tissues, indicating that this peptide distinguishes lymphatics in tumours from normal lymphatics.

Significance of vascular diversity

The functional significance of the vascular diversity established by the phage studies is not well understood. However, membrane dipeptidase, which is selectively expressed in lung vessels, inactivates leukotriene D_4 [26]. It seems possible that this enzymic activity is needed to protect the lungs against systemic pro-inflammatory activity of the cytokine.

The proteins in tumour vasculature, the expression of which is linked to angiogenesis, are probably functionally important in the formation of neovasculature. The growth factor receptors and integrins that are up-regulated in angiogenic vessels are needed for angiogenesis to proceed [1], as is aminopeptidase N [21]. A tumour-homing peptide identified by targeting pancreatic islet cell tumours is homologous with a segment in the PDGF-B precursor protein, and its binding to cultured cells is dependent on the PDGF-β receptor [12]. The peptide appeared to bind both to endothelial cells and pericytes in the tumours. The molecule this peptide binds to probably plays a role in endothelial–pericyte interactions.

Delivery of therapeutic agents to vascular targets

Peptides that home to a specific site in the vasculature are attractive as carriers of therapeutic and diagnostic agents. Homing peptide-directed drug delivery should concentrate the drug at the targeted site, increasing efficacy while decreasing side effects in other tissues. Coupling of doxorubicin with the peptides that specifically bind to the $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins (RGD motif peptide) or to aminopeptidase N (NGR motif peptide) yielded compounds that were more effective and less toxic than doxorubicin alone [7], and similar results were obtained when these peptides were used to target tumour necrosis factor into tumours [27]. Inserting an RGD sequence into an adenovirus surface protein changes the tropism of the virus such that the virus infects cells expressing integrins [28,29]. A non-peptidic compound that binds to $\alpha v\beta 3$ integrin has been used to target a nanoparticle-based gene therapy vector to tumour vasculature [30].

The RGD and NGR peptides could also direct an antibacterial peptide that induces apoptosis in mammalian cells if internalized by the cells. Conjugates of the pro-apoptotic peptide with the integrin and aminopeptidase N binding peptides inhibited tumour growth in mice [31]. Systemic treatment of mice with the integrin-binding conjugate also suppressed inflammation in arthritic synovium, which exhibits strong angiogenesis [32]. Moreover, combining the same pro-apoptotic peptide with a homing peptide that binds to the blood vessels of the normal prostate yielded a compound that caused partial destruction of the normal prostate tissue [14]. Thus the *in vivo* effects of the same non-specifically toxic peptide depended on the specificity of the homing peptide, clearly illustrating the potential of the targeting technology.

Quite recently, we have identified peptides that are capable of being internalized by their target cells and delivering a drug-like payload (fluorescein, rhodamine or biotin) into the cell nucleus [9–12]. These internalizing peptides contain numerous basic amino acid residues, which apparently are important for the internalization and can form a nuclear localization signal. One of these peptides binds to cell-surface nucleolin [24]; the cellular receptors for the other peptides remain to be identified. These internalizing peptides may prove to be particularly useful for delivering anti-cancer drugs that act in the nucleus.

Tissue-specific endothelial molecules in metastasis

It is a commonly held view that much of the tissue bias in metastasis is due to non-specific trapping of circulating tumour cells in the capillaries of the tissues that the cells pass through. This mechanism would explain the frequency of metastasis in the lungs and the liver, which are the tissues that first receive the venous blood. However, there appears to be a specific adhesion component even in metastasis to the lungs. A protein with sequence homology to chloride channels, LuECAM-1, is responsible for the adhesion of B16 murine melanoma cells to lung endothelia [19]. Similarly, binding of a breast cancer cell line to dipeptidyl peptidase IV expressed on lung endothelial cells facilitates the dissemination of these cells to the lungs [33]. These examples indicate that even metastasis to the target organs that are supposed to receive circulating tumour cells is facilitated by specific cell adhesion. Tumours can also show tissue preferences that cannot depend on circulatory routing; again, binding of the tumour cells to selectively expressed endothelial surface molecules seems to be involved [19,33,34].

The identification of a presumed ion channel, protease, and a molecule with unknown function as the receptors for metastasizing tumour cells in the lungs shows that adhesive events resulting in cell homing can depend on molecules

that are distinct from what are usually considered as adhesion molecules. Classical adhesion proteins may also play a role in tissue-specific tumour metastasis. Thus forced expression of the $\alpha4\beta1$ integrin on tumour cells can change the metastatic pattern of the transfected cells; having favoured the lungs as the natural site of metastasis, $\alpha4\beta1$-expressing Chinese-hamster ovary cells and K562 human erythroleukaemia cells metastasized into the lungs and bones from an intravenous injection [35]. Tumour cells that express the Lewis x carbohydrate at their surface more readily metastasize into the lungs compared with control cells lacking Lewis x [36,37]. In this case, the receptor is probably a lectin that recognizes the Lewis x epitope.

We have recently used phage display to identify a new lung-specific adhesion system that promotes lung metastasis by another breast cancer cell line, 4T1 [38]. The adhesion molecule on the tumour cells is a previously uncharacterized membrane protein, and its receptor on lung endothelial cells is not known. Thus our phage display approach probably reveals additional tissue-specific endothelial molecules that are candidate receptors for tissue-specific homing of metastatic tumour cells.

Future directions

Our homing peptide studies indicate that the endothelial cells (and possibly mural cells as well) in each vascular bed express a unique complement of cell-surface molecules. Only a few of the molecules that act as receptors for homing peptides are known, but their diversity (proteases, integrins, growth factor receptors and proteoglycans) suggests that they represent a heterogeneous group of proteins. These proteins are probably functionally significant in the vascular beds that express them. They can also function as tissue-specific receptors for metastatic tumour cells. To accelerate the discovery of homing peptide receptors, we are working on a method that allows simultaneous identification of a homing peptide and its receptor.

The tumour-homing peptides fall into two categories: (i) peptides that bind to angiogenesis-associated molecules and recognize the vasculature of all tumours (and other angiogenic lesions), and (ii) peptides that recognize the vasculature of some, but not all tumours. The latter kind would be potentially advantageous in therapeutic applications because sites of beneficial angiogenesis would not be affected. We are currently screening for peptides that would broadly recognize the vasculature of a given tumour type.

Our demonstration that tumour lymphatics are also specialized offers the possibility of attacking tumours from two directions: through the blood vessels and through the lymphatics. So far, only one homing peptide exists for tumour lymphatics, and it does not recognize all tumours. Screening is in progress to identify more lymphatic homing peptides.

Among the newest homing peptides we have described are several that are internalized by the target cells and can take a payload, such as fluorescein, into the cell nucleus. We are conducting treatment experiments with drug conjugates to utilize these properties. We are also taking advantage of the sensor capabilities of homing peptides in designing nanodevices for tumour diagnosis and treatment.

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