available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Review

Novel challenges in exploring peptide ligands and corresponding tissue-specific endothelial receptors

Maria Luisa Balestrieri[a], *Claudio Napoli*[b,*]

[a]*Department of Chemical Biology and Physics; 1st School of Medicine, II University of Naples, Complesso S. Andrea delle Dame, Naples 80138, Italy*

[b]*Department of General Pathology and Excellence Research Centre on Cardiovascular Diseases, 1st School of Medicine, II University of Naples, Complesso S. Andrea delle Dame, Naples 80138, Italy*

ARTICLE INFO

Article history:
Received 14 December 2006
Received in revised form
6 February 2007
Accepted 8 February 2007

Keywords:
Phage display
Vasculature
Viral vectors
Targeting therapeutic agents

ABSTRACT

The structural and molecular diversity of vascular endothelium may depend on the functional state and tissue localisation of its cells. Tumour vasculature expresses a number of molecular markers that distinguish it from normal vasculature. In cancer, the determinant of specific tumour vasculature heterogeneity is, in part, dictated by dysregulated expression of tumour-derived angiogenic factors. The identification of molecular 'addresses' on the surface of tumour vasculature has significantly contributed to the selection of targets, which have been used for delivering therapeutic and imaging agents in cancer. Cytotoxic drug, pro-apoptotic peptides, protease inhibitors, and gene therapy vectors have been successfully linked to peptides and delivered to tumour sites with an improved experimental therapy. Different diagnostic and therapeutic compounds can be efficiently targeted to specific receptors on vascular endothelial cells; the development of ligand-directed vector tools may promote systemic targeted gene delivery.

Here, we review the very recent advances in the identification of peptide ligands and their corresponding tissue-specific endothelial receptors through the phage display technology with emphasis on ligand-directed delivery of therapeutic agents and targeted gene therapy.

1. Introduction

A dominating feature of tumour vasculature is neoangiogenesis, a multistep process crucial for the progression and metastasis of cancer through the new blood vessel formation from pre-existing vasculature. It is well known that tumour blood vessels differ from the vasculature of normal tissues both structurally and physiologically. Neoformed vessels are discontinuous, leaky and present a dysregulated expression of a number of molecules such as integrins, endothelial cells growth factor receptors, cell surface proteoglycans, proteases, and extracellular matrix components.[1] Targeted therapy provides an efficient means of discriminating between tumour-associated endothelial cells and normal cells and of controlling tumour growth independently from cell type. Tumour growth can be selectively inhibited by blocking tumour-derived angiogenic signals or by directly targeting tumour vascular endothelial cells (Fig. 1). For this purpose, the rapid development of *in vivo* phage display technique allowed the identification of small peptides that target receptors

* *Corresponding author*: Tel./Fax: +39 81 292399.
E-mail address: claunap@tin.it (C. Napoli).
0959-8049/$ - see front matter
doi:10.1016/j.ejca.2007.02.006

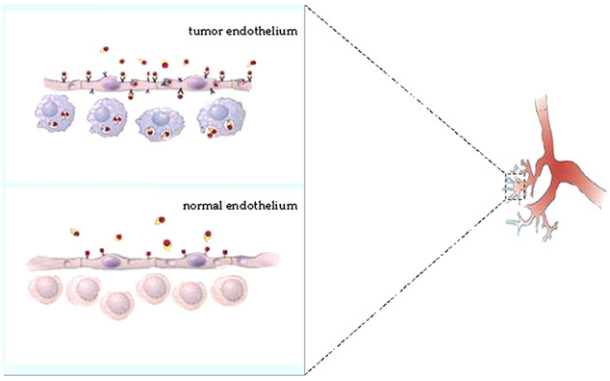

Fig. 1 – Mapping the human vasculature by *in vivo* phage display. The molecular diversity of the luminal endothelial cell surface arising *in vivo* from local variations in genetic expression and tissue microenvironment may create opportunities for achieving targeted molecular imaging and therapies. A random peptide library displayed on bacteriophage is the source of ligands that home to receptors selectively expressed on endothelial cells of normal or tumour endothelium. Ligands isolated by *in vivo* phage display can be used for directing drugs or gene therapy vectors to specific vasculature of pathological tissues.

on endothelial cells.[2] This technique also makes possible to localise novel markers of other diseases where angiogenesis and vascular remodelling occur, such as arthritis, atherosclerosis and diabetic retinopathy.[3–5] Molecular addresses identification on blood vessels is challenging because it involves searching for specific targets on the vasculature highly heterogeneous for molecular and structural diversity.[6,7]

To date, many targeted molecules that are selectively upregulated on angiogenic tumour endothelial cells have been identified. These include integrins, vascular endothelial growth factor (VEGF) receptors, vascular cell adhesion molecules (VCAM), E-selectin, matrix metalloproteinases (MMP-2/MMP-9), amminopeptidase N (APN), amminopeptidase A (APA), amminopeptidase P (APP), endoglin (CD105), and molecules associated with procoagulant changes on angiogenic endothelium such as phosphatidylserine.[8,9]

Technological innovation and novel applications advanced the field of *in vivo* phage display. However, to ensure safety, efficacy, and specificity of ligand-directed vascular delivery preclinical or clinical investigation are still required.

Here, we did not provide a simple update of the literature in the field. Since there are ongoing clinical trials that are evaluating some therapeutical approaches based on this scientific background we attempt a framework of a pure example of translational research in pathobiology and treatment of advanced malignancy and vascular damage.

2. Identification of vascular addresses for ligand-based vascular targeting

Ligand-directed vascular targeting can be accomplished by specific peptides, antibodies,[10] and growth factors complexed with selective effector molecules.[11]

The identification of homing motifs is achieved by screening peptide libraries *in vitro* on cells, *in vivo*, or *ex-vivo* on surgical dissected specimens. *In vivo* phage display technology is mainly performed on mice and only one example of screening on human patients is currently available.[12] The success of phage display selection depends primarily upon the quality of the library used, as ligands that are not represented cannot be isolated. Phage libraries are comprised of millions of polypeptides expressed within the coat proteins of filamentous bacteriophages, protein 3 (pIII), protein 6 (pVI) or protein 8 (pVIII) and their construction and use has been extensively reviewed.[13,14] Epitopes of interest can be targeted with these foreign proteins expressed on the phage and those phage particles that bind to the epitope can be isolated, transduced back into the bacteria and the bacteria grown to expand the selected phage population, as extensively reviewed elsewhere.[2]

In the *in vivo* phage-display screening for peptide that home to human tissues, phage libraries are injected intravenously to identify phage peptides that interact with molecules specifically expressed on the blood vessels of a particular organ or tumour (Fig. 2). After removal of whole organs, tumours, or tissue biopsies, bound phages are eluted

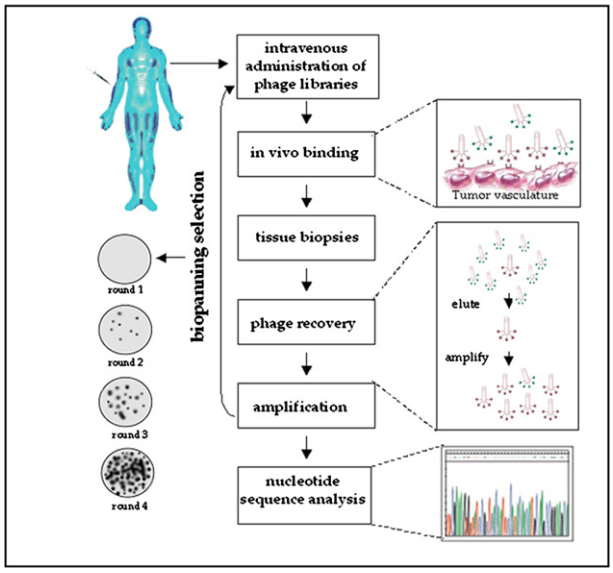

Fig. 2 – *In vivo* phage display technology developed to isolate peptides that home selectively to intact specific targets expressed on human blood vessels. Phage libraries are injected intravenously to identify phage peptides that interact with molecules specifically expressed on blood vessels of a particular organ or tumour. After removal of whole organs, tumours, or tissue biopsies, bound phage are eluted and amplified by growth in host bacteria. Amplification is followed by biopanning, multiple rounds of injection of phage, vascular perfusion, tissue removal, phage isolation, and amplification, until a population of phages that selectively bind to blood vessels in the organ of interest is obtained. The DNA corresponding to the insert is then sequenced within the phage genome, allowing the insert to be reproduced as a synthetic peptide outside the context of the phage particle.

and amplified by growth in host bacteria. The amplification process is highly efficient because the phages infect and propagate in bacteriophages which secrete multiple copies. Amplification is followed by biopanning, multiple rounds of injection of phage, vascular perfusion, tissue removal, phage isolation, and amplification, until a population of phages that selectively bind to blood vessels in the organ of interest is obtained. The DNA corresponding to the insert is then sequenced within the phage genome, allowing the insert to be reproduced as a synthetic peptide outside the context of the phage particle (Fig. 2).

Using *in vivo* phage display technology with murine tumourigenesis models, two tumour peptides homing blood vessels, one identical to a sequence of kallikrein-9[15] and one omologous to pro-PDGF-B[16] were identified and found to be tumour stage-specific. Although isolation of peptide probes in murine models may present hurdles associated with translation of data to humans, these studies provide a temporal pattern of selective phage homing during tumour progression from premalignant to invasive phase.

In addition to peptides, several single chain antibodies (scFv) can be displayed on the surface of phage particles, and the sequence that binds most efficiently can be selected by biopanning.[17]

A consistent number of studies describe the isolation of antibody molecules against receptors or adhesion molecules expressed by endothelial cells. These include the selection of antibodies against VEGF receptor[18] and the selection of scFv fragments directed against VEGF-VEGFR complex,[19] CD105,[17,20] angiopoietin-2 (Ang-2),[21] laminin,[22] ErbB2,[23] and against new targets for tumour vasculature, such as Etk kinase,[24] plexin,[25] and TEM1.[26]

To date, despite the technical difficulties, phage display method allowed the identification of several tissue-specific and angiogenesis-related vascular ligands.[12,27] A recent advanced application of this technique is shown by Kolonin and colleagues [28] describing the simultaneous screening of phage display libraries for peptides homing to a number of tissues without the need for an individual subject for each target tissue. This has been tested in mice by selecting homing peptides for six single organs in a single screen and prioritising them by using software compiled for statistical validation of peptide biodistribution specificity. Possible applications in patient studies would confirm the usefulness of this approach.

A major challenge for the vascular targeting field is mapping, screening, and validating potential targets and their probes under native conditions found *in vivo* since the extensive and quite rapid loss of normal morphology and protein expression by endothelial cells *ex vivo* can drastically limit their utility in target-discovery efforts.[29] However, in the last decade, the yield of promising tissue-specific targets identified by screening of phage libraries after intravenous injection has been rather modest, probably due to several limitations. These include (i) the additional complexity introduced by substantial convective forces from blood flow on phage binding and retention at the luminal endothelial cell surface *in vivo*, (ii) the inability to block *a priori* high nonspecific binding of phage, and (iii) the unwarranted randomness in the assay from the short contact time and incomplete exposure of the phage-display diversity to the endothelial cell surface of each organ, because nearly all of the intravenous injected phages are scavenged within minutes or less by the liver and spleen.[30]

A recent example for harnessing the power of phage display for mapping vascular endothelia natively in tissue and for achieving vascular targeting of specific tissues is represented by the use of phage-displayed antibodies converted into single chain antibodies (scFv)-Fc fusion proteins to rapidly target selected organs *in vivo* preserving native protein expression *in vivo* and obviating liver uptake of intravenously injected phage.[7] Furthermore, advances in proteomic technologies allowed the identification of accessible biomarkers in human tissues thorough *in vivo* or *ex vivo* biotinylation of protein combined to mass spectrometry.[31] These novel approaches may complement previous robust technologies to explore more comprehensively endothelial molecular diversity *in vivo*.

3. Therapeutic application of ligand-based vascular targeting

The identification of specific ligand-receptor pairs and the knowledge of their cellular distribution and accessibility represent a crucial step in the development of targeted pharmaceuticals.[32,33] To date, several functionally relevant homing peptides have been identified in tumours, primarly in mice and also in patients,[34–36] as well as peptides probes that selectively bind to atherosclerotic lesions[37,38] (Table 1, Refs[8,15,16,39–47]). They can be used to selectively target vasculature with radiolabelled,[48] cytotoxic agents, antiangiogenic, procoagulant, immunomodulatory molecules,[49] or with gene therapy vectors.[50]

A recent interesting application of RGD-4C peptide is described by Yao and colleagues[51] who studied the distribution of RGD-4C phage on tumour blood vessels before and after antiangiogenic therapy. The reduction in integrin expression on tumour vessels after antiangiogenic therapy raises the possibility that integrin-targeted delivery of diagnostics or therapeutics may be compromised. They observed that the distribution of phage was similar to alpha(v)beta(3) and alpha(5)beta(1) integrin expression. Blood vessels that survived treatment with AG-013736, a small molecule inhibitor of VEGF and platelet-derived growth factor receptors (PDGF), had only 4% as much binding of RGD-4C phage compared with vessels in untreated tumours. Cellular distribution of RGD-4C phage in surviving tumour vessels matched the alpha(5)beta(1) integrin expression.

When coupled to the anticancer drug doxorubicin, RGD-4C peptide enhanced the efficacy of the drug against human breast cancer xenografts in nude mice and also reduced its toxicity.[52] Based on these results, delivery of drugs might benefit from identification by *in vivo* phage display of targeting peptides that bind to tumour blood vessels normalised by antiangiogenic agents. However, quantitative analysis of the RGD-4C biodistribution to validate this peptide as a portable tumour vascular targeting agent are not fully described and therapeutic results are not always reproducible.

Tumour vascular targeting was successfully achieved by coupling IFN to GCNGRC, a CD13 ligand, by genetic engineer-

Table 1 – Molecular addresses on vascular endothelia isolated by in vivo phage display

Target	Function	Localisation	Peptide sequence	Ref.
αv Integrins	cell adhesion	normal/breast tumour endothelium	CDCRGDCFC	39
APN/CD13	protease	normal/breast tumour endothelium, perycites	CNGRC	40
APA	protease	tumour endothelium, perycites, stromal cells	CPRECESIC	41
APP	protease	breast tumour endothelium	CPGPEGAGC	42
MMP2	protease	normal/tumour endothelium	CTTHWGFTLC	8
MMP9	protease	normal/tumour endothelium	CRRHWGFEFC	8
NG2	proteoglycan	tumour endothelium	GLS	42
IL11-r	cytokine receptor	prostate tumour endothelium	CGRRAGGSC	43
Prohibitin	membrane chaperone	white fat vasculature	CVPELGHEC	44
KDR/Flk-1	VEGF receptor	HUVEC, tumour endothelium	HTMYYHHYQHHL	45,46
VCAM-1	cell adhesion	tumour endothelium	VHSPNKK	47
Kallikrein-9 substrate	protease substrate	tumour endothelium	CSRPRRSEC	15
PDGFR	PDGF receptor	premalignant angiogenic islet	CRGRRST	16

ing technology. Targeted delivery of low doses of IFN to CD13, a marker of angiogenic vessels, can overcome major counter-regulatory mechanisms and delay tumour growth in two murine models that respond poorly to IFN.[53]

It has been also possible to design growth factor-toxin or adhesion molecule-toxin conjugates with the aim of restricting tumour's blood supply. For example, tumour tissue necrosis and intratumour vascular fibrin thrombosis were observed after a single systemic administration of a novel single-chain anti-VCAM-1 antibody/soluble tissue factor (TF) fusion protein as a tumour vascular targeting agent.[47] Interestingly, no evidence of treatment-related vascular thrombosis, necrosis, in normal tissues were observed validating the notion that strategies aimed at producing vascular occlusion in tumour tissue can be selective.

A good example of recombinant fusion proteins is represented by three fusion proteins with truncated TF (tTF), i.e. chTNT-3/tTF, chTV-1/tTF, and RGD/tTF.[54] The first fusion protein, chTNT-3/tTF, targets extracellular DNA which may accumulate on the endothelial cell surface or the basement membrane in necrotic regions of the tumour. The second fusion protein, chTV-1/tTF, targets a vessel antigen, fibronectin, which is located in the basement membrane of vessels but only accessible in fenestrated (leaky) tumour endothelium. The third fusion protein, RGD/tTF, targets endothelial $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins exposed in tumour vessels of several tumour types. Of interest, the chTV-1/tTF and RGD/tTF fusion proteins induced thrombosis in small and medium sized tumour vessels, whereas the chTNT-3/tTF induced clotting in relatively larger vessels.[54] Combining all 'coaguligands' would be the most effective treatment strategy to inhibit tumour outgrowth demonstrating the possibility of controlling the vascular diversity of endothelium during tumour progression with a multi-target approach.

Current developments of targeted antiangiogenic agents include their use in combined therapy, including targeted antivascular agents and conventional chemoterapeutic agents. A novel system to increase the efficacy of combined therapy has been recently demonstrated with a 'nanocell' system made of a nuclear nanoparticle within an extranuclear lipid envelope.[55] This system, which enables a first temporal release of vascular targeting agent followed by the release of a chemotherapeutic drug from the inner nanoparticle, seems to posses an antitumour activity higher than the direct administration of equivalent doses.[55] However, the lack of whole-body imaging and of a careful biodistribution analysis, important complements to ascertain targeting, prevents a final evaluation of potentials and problems associated with the use of this technology.

Clinical trial experiences on the development of growth factor receptor-targeted antiangiogenic approaches for advanced cancer or on the delivery of non-viral gene therapy for therapeutic angiogenesis in humans with disabling myocardial ischemia and peripheral vascular disease are promising.[56,57] However, the identification of markers that can monitor activity and efficacy of antiangiogenic drugs in patients belongs to the most critical challenges to exploit the full potential of antiangiogenic therapies.

Ligands isolated by *in vivo* phage are able to exert an antiproliferative or proapoptotic effect either by vascular targeting action or by a direct cytotoxic activity against tumour cells. As example, a novel 10-amino peptide containing the RGD sequence shows direct tumour cytotoxic and invasiveness inhibition effects dependent on the blockage of alpha(v) integrin activity.[58] This RGD peptide, GCGGRGDGGC, inhibited tumour cell proliferation and also induced apoptosis and G1-phase cell cycle arrest in cell lines that bound and internalised the peptide. Similarly, a novel peptide, AV3, contained in the NH2 terminus of VEGF receptor-3[59] has been recently reported to couple to a TAT cellular penetration sequence and to cause specific displacement of Focal Adhesion Kinase (FAK) from the focal adhesion and affects co-localisation of FAK and VEGF receptor. AV3 peptide also reduced proliferation and induced apoptosis in breast cancer cell line.[59]

Phase I trials are ongoing on the efficacy and safety of ligand-directed vascular targeting. NGR-TNF, a derivative of TNF-alpha, consists of TNF fused to CNGRCG. Previous studies showed that NGR-TNF can exert synergistic antitumour effects with doxorubicin and with other chemotherapeutic drugs in murine models although it does not exhibit highly selective accumulation at the tumour site.[60,61]

The NGR-TNF represents an important case study in this field as it being evaluated in the clinic as a possible strategy for increasing the therapeutic index of chemotherapeutic

drugs. An ongoing clinical trial is testing the side effects and best dose of NGR-TNF in treating patients with advanced solid tumours (Clinical Trials Gov. Identifier: NCT00098943). Patients of this trial receive NGR-TNF IV over 20–60 min on day 1. Courses repeat every 21 days in the absence of unacceptable toxicity or disease progression. Cohorts of one to six patients receive escalating doses of NGR-TNF until the maximum tolerated dose (MTD) is determined. The MTD is defined as the dose preceding that at which two of six patients experience dose-limiting toxicity. Up to 12 patients receive treatment at the MTD. Diagnostic application of peptide or antibodies is also tested in a study of imaging colo-rectal cancer using a two step antibody technique in nuclear imaging (Clinical Trials.gov Identifier: NCT00185081). The aim of this trial is to bypass the slow solid tumour uptake of antibodies by the use of an antibody pre-targeting method and an in labelled peptide, IMP-205. The hypothesis of this study is that this small peptide will be readily absorbed by the tumour which is pre-saturated by antibodies. As a consequence a higher radiation dose can be given with a lower toxicity. Dose-limiting toxicity results to apply this approach on large scale in the future are expected.

4. Vascular targeted gene therapy

Vascular targeting of gene delivery can be accomplished by using viral gene vectors or non viral gene vectors.[8,62,63] This is based on the unique properties of viral vectors to be easily manufactured, engineered to selectively express genes in tumour endothelial cells for long periods of time or to destroy endothelial cells once they bind.[62] Advantages of the application of viral vectors, adenovirus (Ad),[64] adeno-associated viral vectors (AAV),[65] retroviral vectors (RV),[66] lentiviral vectors (LV), measles viral vectors (MV), and herpes simplex viral vectors (MSV)[67] include the combinationof antiangiogenesis and tumour vascular targeting therapy with other strategies.

Gene therapy delivery devices targeted by using phage display-derived peptides, like peptides containing RGD-polyethylenimine (PEI) conjugates, efficiently bind DNA and enhance its transfer to different cell types.[68] The cyclic RGD-4C conjugated to liposomes has been used as a delivery device to endothelial and melanoma cells expressing $\alpha v\beta 3$ integrins. Systemic administration of nanoparticles complexed to $\alpha v\beta 3$ integrin-ligand to deliver a mutant raf to tumour-bearing mice resulted in apoptosis of the endothelium leading to regression of metastatic tumours.[69] Tumour growth was inhibited by a similar strategy using small interfering RNA, specifically knocking down VEGF receptor 2 expression.[70]

Several endothelial promoters have been used in the transcriptionally targeted viral vectors to tumour cells, including that of the VEGF receptor Flt-1,[71,72] ICAM-2, and the murine preproendothelin-1 (PPE-1) promoter.[73] Treatment of xenograft tumour models with RV in which the herpes simplex virus thymidine kinase *(HSV-TK)* gene was driven by a hybrid endothelial-specific PPE-1 long terminal repeat (LTR) resulted in widespread vascular disruption and apoptosis when combined with chemotherapeutic agents.[74] AAV2 and AAV5 mediate delivery of antiangiogenesis factors, such as a truncated form of the VEGF receptor Flk-1, or antisense mRNA against VEGF.[75,76]

Local delivery of the Ad vector Ad-hVEGFR2-iCaspase-9 followed by intraperitoneal injection of a cell-permeable drug AP20187 resulted in endothelial cell apoptosis and local ablation of microvessels in the severe combined immunodeficient (SCID) mouse model of angiogenesis.[77] Another approach would be the delivery of Adp21/WAF/CIP to promote vascular apoptosis in restenotic arteries.[78] Hedley and colleagues [79] have re-engineered the Ad fiber protein so it can accommodate scFv antibodies. Specifically, they have engineered a framework for scFv that is stable in cytosol and selected complementarity determining regions (CDRs) of the heavy and light chains of the scFv, which are thermodynamically stable and fold correctly in the cytosol. These technological breakthroughs have enabled the targeting of Ad vectors to the vascular endothelial cells with scFv on fibre without destabilising the packing of the vector.

Recently, a single administration of the AdfVII/IgG(1)Fc vector has been shown to destroy the peripheral but not the central vasculature of mouse breast tumour spheroids, causing partial tumour regression.[80] Subcutaneous injection of the AdfVII/IgG(1)Fc vector leads to the release into the system circulation of a fVII/IgG(1)Fc immunoconjugate molecule that binds specifically and tightly to TF on vascular endothelial cells and tumour cells, activating a cytolytic immune response against the targeted cells.[80] Additional administrations of AdfVII/IgG(1)Fc prevented regeneration of the peripheral vasculature and regrowth of the tumour indicating that a critical parameter for optimising tumour damage is the schedule for successive administrations of the AdfVII/IgG(1)Fc, which should coincide with the regeneration of the peripheral vasculature and continue until the tumour is destroyed.[80]

Safety and efficacy concerns associated with gene therapy vectors can be overcome by ablating their endogenous undesired tropism and retargeting them to a specific tissue.[62] AAV tropism has been genetically engineered by the incorporation of endothelial cell-targeted peptides discovered by phage display into the capsids of the vector.[81–83] Peptide-modified AAV has been shown to possess enhanced transduction of human endothelial cells compared with AAV with a wild-type capsid.[81–83] The identification of four novel consensus peptides homing to the lung or brain was achieved by extensive biopanning in rats.[66] Each peptide incorporated into the VP3 region of the AAV-2 capsid to display the peptide at the virion surface allowed *in vivo* homing to the endothelium residing in defined organs.[66] A very recent approach was based on Ad delivery of MMP inhibitor coupled with BMC in experimental stroke.[84]

To retarget viral vector to cell type specific receptors, the incorporation of peptides selected by phage display into the viral capsid might, sometimes, present a low ligand-receptor binding affinity due to changes of peptide conformation in the virus protein context. A peptide screening system displayed directly on AAV2 leading to the isolation of AAV vectors selective for endothelial cells *in vitro* was reported by Muller and colleagues.[85] This library was generated in a tree-step system that ensures encoding of displayed peptides by the packaged DNA. The selected peptides enhanced trans-

duction in coronary cells but not in non endothelial cells as control and overcame the limitation of phage display selection.

Although vectors engineered to bind and to infect tumour vascular endothelial cells and tumour cells work *in vitro*, only very limited targeting is seen *in vivo*. Several reasons account for this, like the low efficiency of delivery to the tumour, the lack of selectivity between tumours and other organs, the uptake by reticuloendothelial system (RES) and hepatic cells, the neutralising antibodies to the vector, and side effects (local inflammation, fever).

Ways to circumvent the uptake of viral vectors by RES and hepatic cells, especially the Ad vector, include the use of substances inhibiting the function of the RES,[86] of antibodies to retarget the virus to alternative receptors such as angiotensin-converting enzyme (ACE),[87] or of targeting peptides inserted into the HI loop of the fiber structure.[65] Hallak and colleagues[88] described a way to circumvent the neutralising antibodies for MV through engineering new targeting ligands into the virus. To target activated endothelial cells in tumour tissue, they developed an Echistatin-targeted MV vector (MV-ERV) which binds to the $\alpha v\beta 3$ integrin receptor with high affinity. MV-ERV vector can also target to tumour cells by the H protein binding to the native CD46 receptor demonstrating its potential use in gene therapy not only for targeting tumour-associated vasculature but also for the treatment of solid tumours.

Clinical trials to test safety and efficacy of targeted gene transfer in colorectal cancer metastatic to liver (Clinical Trials.gov Identifier: NCT00035919) or to determine the antiangiogenic efficacy of adenoviral endostatin in the treatment of advanced solid tumour (Clinical Trials.gov Identifier: NCT00262327) are still ongoing. For more details about the Clinical Trials.gov mentioned in this review, please, visit the web site http://nccam.nih.gov/clinicaltrials.

5. Take 'home' messages and the road ahead

In the last decade, *in vivo* phage display technology has yielded homing peptides and antibodies that have revealed the high heterogeneity of the vasculature. The identification of vascular novel addresses on blood vessels has increased the development of therapeutic strategies which, together with therapeutic angiogenesis during tissue ischemia elicited by bone marrow cell therapy,[89] opened up possibilities for the treatment of cancer and vascular diseases, including pulmonary hypertension.[90]

The fact that tumour blood vessels differ from those in normal organs and from newly formed blood vessels in healing wounds, chronically inflamed tissues and other sites of angiogenesis, creates the opportunity for identifying truly tumour specific vascular markers. The extraordinary complex network regulating abnormal expression of genes that contribute to the pathophysiological and clinical manifestations of tumour-associated vasculature[91] provides candidate targets for tumour-selective 'transductional' and 'transcriptional' targeting of genetically or chemically modified vectors.[80] However, an obstacle can be represented by the fact that vectors tested in mouse models for their suitability in gene therapy targeting to tumour endothelium may not be totally predictive of the outcome in clinical trials in human subjects.

Therapeutic ligand-based vascular targeting agents are proving their usefulness through the ongoing clinical trials in humans, and outcomes, both in terms of therapeutic gain and vector-associated toxicity issues, are keenly awaited. Although combined therapy, including targeted antiangiogenic agents and conventional tumour therapy, renewed the confidence in targeted approaches, modes of action and dose dependence are still open issues for safe and efficacious applications. The real goal is to find some way in combined therapy of inducing a synergistic therapeutic effect rather than a pure additive beneficial result. A major detractor to the successful application of gene therapy for the treatment of a range of diseases is not a paucity of therapeutic genes but the lack of an efficient non-toxic gene delivery system in chronic long-lasting therapy. Future efforts will be indispensable in developing safe and widely distributed viral vectors and gaining in depth insight into metastatic tumour models in which very poor therapeutic approaches are available.

Conflict of interest statement

None declared.

REFERENCES

1. Bussolino F, Mantovani A, Persico MG. Molecular mechanisms of blood vessel formation. *Trends Biochem Sci* 1997;**22**:251–6.
2. Zurita AJ, Arap W, Pasqualini R. Mapping tumor vascular diversity by screening phage display libraries. *J Control Release* 2003;**91**:183–6.
3. Fuster V. Mechanisms leading to myocardial infarction: insights from studies of vascular biology. *Circulation* 1994;**90**:2126–46.
4. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1995;**1**:27–31.
5. Witmer AN, Vrensen GF, Van Noorden CJ, Schlingemann RO. Vascular endothelial growth factors and angiogenesis in eye disease. *Prog Retin Eye Res* 2003;**22**:1–29.
6. Pasqualini R, Arap W, McDonald D. Probing the structural and molecular diversity of tumor vasculature. *Trends Mol Med* 2002;**8**:563–71.
7. Oh Y, Mohiuddin I, Sun Y, et al. Phenotypic diversity of the lung vasculature in experimental models of metastases. *Chest* 2005;**128**:596S–600S.
8. Hajitou A, Pasqualini R, Arap W. Vascular Targeting: recent advances and therapeutic perspectives. *Trends Cardiovasc Med* 2006;**16**:80–8.
9. Liu Y, Deisseroth A. Tumor vascular targeting therapy with viral vectors. *Blood* 2006;**107**:3027–33.
10. Mutuberria R, Satijn S, Huijbers A, et al. Isolation of human antibodies to tumor-associated endothelial cell markers by in vitro human endothelial cell selection with phage display libraries. *J Immunol Methods* 2004;**287**:31–47.
11. Thorpe PE. Vascular targeting agents as cancer therapeutics. *Clin Cancer Res* 2004;**10**:415–27.
12. Arap W, Kolonin MG, Trepel M, et al. Steps toward mapping the human vasculature by phage display. *Nat Med* 2002;**8**:121–7.
13. Romanov VI. Phage display selection and evaluation of cancer drug targets. *Curr Cancer Drug Targets* 2003;**3**:119–29.

14. Brown KC. New approaches for cell-specific targeting: identification of cell-selective peptides from combinatorial libraries. *Curr Opin Chem Biol* 2000;**4**:16–21.
15. Hoffman JA, Giraudo E, Singh M, et al. Progressive vascular changes in a transgenic mouse model of squamous cell carcinoma. *Cancer Cell* 2003;**4**:383–91.
16. Joyce JA, Laakkonen P, Bernasconi M, Bergers G, Ruoslahti E, Hanahan D. Stage-specific vascular markers revealed by phage display in a mouse model of pancreatic islet tumorigenesis. *Cancer Cell* 2003;**4**:393–403.
17. Volkel T, Muller R, Kontermann RE. Isolation of endothelial cell-specific human antibodies from a novel fully synthetic scFv library. *Biochem Biophys Res Commun* 2004;**317**:515–21.
18. Lu D, Shen J, Vil MD, et al. Tailoring in vitro selection for a picomolar affinity human antibody directed against vascular endothelial growth factor receptor 2 for enhanced neutralizing activity. *J Biol Chem* 2003;**278**:43496–507.
19. Miao HQ, Hu K, Jimenez X, et al. Potent neutralization of VEGF biological activities with a fully human antibody Fab fragment directed against VEGF receptor 2. *Biochem Biophys Res Commun* 2006;**345**:438–45.
20. Volkel T, Holig P, Merdan T, Muller R, Kontermann RE. Targeting of immunoliposomes to endothelial cells using a single-chain Fv fragment directed against human endoglin (CD105). *Biochim Biophys Acta* 2004;**1663**:158–66.
21. Cai M, Zhang H, Hui R. Single chain Fv antibody against angiopoietin-2 inhibits VEGF-induced endothelial cell proliferation and migration in vitro. *Biochem Biophys Res Commun* 2003;**309**:946–51.
22. Sanz L, Kristensen P, Blanco B, et al. Single-chain antibody-based gene therapy: inhibition of tumor growth by in situ production of phage-derived human antibody fragments blocking functionally active sites of cell-associated matrices. *Gene Ther* 2002;**9**:1049–53.
23. Mazuet C, Lerouge D, Poul MA, Blin N. Breast carcinoma specific antibody selection combining phage display and immunomagnetic cell sorting. *Biochem Biophys Res Commun* 2006;**348**:550–9.
24. Paz K, Brennan LA, Iacolina M, Doody J, Hadari YR, Zhu Z. Human single-domain neutralizing intrabodies directed against Etk kinase: a novel approach to impair cellular transformation. *Mol Cancer Ther* 2005;**4**:1801–9.
25. Roodink I, Raats J, van der Zwaag B, et al. Plexin D1 expression is induced on tumor vasculature and tumor cells: a novel target for diagnosis and therapy? *Cancer Res* 2005;**65**:8317–23.
26. Marty C, Langer-Machova Z, Sigrist S, Schott H, Schwendener K, Ballmer-Hofer K. Isolation and characterization of a scFv antibody specific for tumor endothelial marker 1 (TEM1), a new reagent for targeted tumor therapy. *Cancer Lett* 2006;**235**:298–308.
27. Smith J, Kontermann RE, Embleton J, Kumar S. Antibody phage display technologies with special reference to angiogenesis. *FASEB J* 2005;**19**:331–41.
28. Kolonin MG, Sun J, Do KA, et al. Synchronous selection of homing peptides for multiple tissues by in vivo phage display. *FASEB J.* 2006;**7**:979–81.
29. Durr E, Yu J, Krasinska KM, et al. Direct proteomic mapping of the lung microvascular endothelial cell surface in vivo and in cell culture. *Nat Biotechnol* 2004;**22**:985–92.
30. Valadon P, Garnett JD, Testa JE, Bauerle M, Oh P, Schnitzer JE. Screening phage display libraries for organ-specific vascular immunotargeting in vivo. *Proc Natl Acad Sci USA* 2006;**103**:407–12.
31. Castronovo V, Waltregny D, Kischel P, et al. Chemical Proteomics Approach for the Identification of Accessible Antigens Expressed in Human Kidney Cancer. *Mol Cell Proteomics* 2006;**5**:2083–91.
32. Trepel M, Wadih A, Pasqualini R. In vivo phage display and vascular heterogeneity: implications for targeted medicine. *Curr Opin Chem Biol* 2002;**6**:399–404.
33. Napoli C, Lerman LO, Sica V, Lerman A, Tajana G, de Nigris F. Microarray analysis: a novel research tool for cardiovascular scientists and physicians. *Heart* 2003;**89**:597–604.
34. Ruoslahti E. Vascular zip codes in angiogenesis and metastasis. *Biochem Soc Trans* 2004;**32**:397–402.
35. Krag DN, Shukla GS, Shen GP, et al. Selection of tumor-binding ligands in cancer patients with phage display libraries. *Cancer Res* 2006;**66**:7724–33.
36. Liang S, Lin T, Ding J, et al. Screening and identification of vascular-endothelial-cell-specific binding peptide in gastric cancer. *J Mol Med* 2006;**84**:764–73.
37. Liu C, Bhattacharjee G, Boisvert W, Dilley R, Edgington T. In vivo interrogation of the molecular display of atherosclerotic lesion surfaces. *Am J Pathol* 2003;**163**:1859–71.
38. Kelly KA, Nahrendorf M, Yu AM, Reynolds F, Weissleder R. In vivo phage display selection yields atherosclerotic plaque targeted peptides for imaging. *Mol Imaging Biol* 2006;**8**:201–7.
39. Dickerson EB, Akhtar N, Steinberg H, et al. Enhancement of the antiangiogenic activity of interleukin-12 by peptide targeted delivery of the cytokine to $\alpha v\beta 3$ integrin. *Mol Cancer Res* 2004;**2**:663–73.
40. Curnis F, Sacchi A, Borgna L, Magni F, Gasparri A, Corti A. Enhancement of tumor necrosis factor a antitumor immunotherapeutic properties by targeted delivery to aminopeptidase N (CD13). *Nat Biotechnol* 2000;**18**:1185–90.
41. Marchiò S, Lahdenranta J, Schlingemann RO. Aminopeptidase A is a functional target in angiogenic blood vessels. *Cancer Cell* 2004;**5**:151–62.
42. Burg MA, Pasqualini R, Arap W. NG2 proteoglycan-binding peptides target tumor neovasculature. *Cancer Res* 1999;**59**:2869–74.
43. Zurita AJ, Troncoso P, Cardo-Vila M, Logothetis CJ, Pasqualini W, Arap W. Combinatorial screenings in patients: the interleukin-11 receptor alpha as a candidate target in the progression of human prostate cancer. *Cancer Res* 2004;**64**:435–9.
44. Kolonin MG, Saha PK, Chan L, Pasqualini R, Arap W. Reversal of obesity by targeted ablation of adipose tissue. *Nat Med* 2004;**10**:625–32.
45. Hetian L, Ping A, Shumei S, et al. A novel peptide isolated from a phage display library inhibits tumor growth and metastasis by blocking the binding of vascular endothelial growth factor to its kinase domain receptor. *J Biol Chem* 2002;**277**:43137–42.
46. El-Mousawi M, Tchistiakova L, Yurchenko L, et al. A vascular endothelial growth factor high affinity receptor 1-specific peptide with antiangiogenic activity identified using a phage display peptide library. *J Biol Chem* 2003;**278**:46681–91.
47. Dienst A, Grunow A, Unruh M, et al. Specific occlusion of murine and human tumor vasculature by VCAM-1-targeted recombinant fusion proteins. *J Natl Cancer Inst* 2005;**97**: 733–47.
48. Kelly KA, Allport JR, Tsourkas A, Shinde-Patil VR, Josephson L, Weissleder R. Detection of vascular adhesion molecule-1 expression using a novel multimodal nanoparticle. *Circ Res* 2005;**96**:327–36.
49. Halin C, Rondini S, Nilsson F, et al. Enhancement of the antitumor activity of interleukin-12 by targeted delivery to neovasculature. *Nat Biotechnol* 2002;**20**:264–9.
50. Parker AL, Fisher KD, Oupicky D, et al. Enhanced gene transfer activity of peptide-targeted gene-delivery vectors. *J Drug Target* 2005;**13**:39–51.

51. Yao VJ, Ozawa MG, Varner AS, et al. Antiangiogenic therapy decreases integrin expression in normalized tumor blood vessels. *Cancer Res* 2006;**66**:2639–49.
52. Arap W, Pasqualini R, Ruoslahti E. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. *Science* 1998;**279**:377–80.
53. Curnis F, Gasparri A, Sacchi A, Cattaneo A, Magni F, Corti A. Targeted delivery of IFN gamma to tumor vessels uncouples antitumor from counterregulatory mechanisms. *Cancer Res* 2005;**65**:2906–13.
54. Hu P, Yan J, Sharifi J, et al. Comparison of three different targeted tissue factor fusion proteins for inducing tumor vessel thrombosis. *Cancer Res* 2003;**63**:5046–53.
55. Sengupta S, Eavarone D, Capila I, et al. Temporal targeting of tumour cells and neovasculature with a nanoscale delivery system. *Nature* 2005;**436**:568–72.
56. Beerepoot LV, Radema SA, Witteveen EO, et al. Phase I clinical evaluation of weekly administration of the novel vascular-targeting agent, ZD6126, in patients with solid tumors. *J Clin Oncol* 2006;**24**:1491–8.
57. Shah PB, Losordo DW. Non-viral vectors for gene therapy: clinical trials in cardiovascular disease. *Adv Genet* 2005;**54**:339–61.
58. Yang W, Meng L, Wang H, et al. Inhibition of proliferative and invasive capacities of breast cancer cells by arginine-glycine-aspartic acid peptide in vitro. *Oncol Rep* 2006;**15**: 113–7.
59. Garces CA, Kurenova EV, Golubovskaya VM, Cance WG. Vascular endothelial growth factor receptor-3 and focal adhesion kinase bind and suppress apoptosis in breast cancer cells. *Cancer Res* 2006;**66**:1446–14454.
60. Corti A, Ponzoni M. Tumor vascular targeting with tumor necrosis factor alpha and chemotherapeutic drugs. *Ann N Y Acad Sci* 2004;**1028**:104–12.
61. Sacchi A, Gasparri A, Curnis F, Bellone M, Corti A. Crucial role for interferon gamma in the synergism between tumor vasculature-targeted tumor necrosis factor alpha (NGR-TNF) and doxorubicin. *Cancer Res* 2004;**64**:7150–5.
62. George SJ, de Nigris F, Baker AH, Napoli C. Gene therapy for vascular diseases. *Gene Therapy Mol Biol* 2003;**7**:135–51.
63. Bazan-Peregrino M, Seymour LW, Harris AL. Gene therapy targeting to tumor endothelium. *Cancer Gene Ther* 2007;**14**:117–27.
64. Bouquet C, Lamande N, Brand M, et al. Suppression of angiogenesis, tumor growth, and metastasis by adenovirus-mediated gene transfer of human angiotensinogen. *Mol Ther* 2006;**14**:175–82.
65. Nicklin SA, White SJ, Nicol CG, Von Seggern DJ, Baker AH. In vitro and in vivo characterisation of endothelial cell selective adenoviral vectors. *J Gene Med* 2004;**6**:300–8.
66. Work LM, Buning H, Hunt E, et al. Vascular bed-targeted in vivo gene delivery using tropism-modified adeno-associated viruses. *Mol Ther* 2006;**13**:683–93.
67. Wong RJ, Chan MK, Yu Z, et al. Angiogenesis inhibition by an oncolytic herpes virus expressing interleukin 12. *Clin Cancer Res* 2004;**10**:4509–16.
68. Kunath K, Merdan T, Hegener O. Integrin targeting using RGD-PEI conjugates for in vitro gene transfer. *J Gene Med* 2003;**5**:588–99.
69. Hood JD, Bednarski M, Frausto R, et al. Tumor regression by targeted gene delivery to the neovasculature. *Science* 2002;**296**:2404–7.
70. Schiffelers RM, Ansari A, Xu J, et al. Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. *Nucleic Acid Res* 2004;**32**:e149.
71. Kaliberov SA, Kaliberova LN, Stockard CR, Grizzle WE, Buchsbaum DJ. Adenovirus-mediated FLT1-targeted proapoptotic gene therapy of human prostate cancer. *Mol Ther* 2004;**10**:1059–70.
72. Richardson TB, Kaspers J, Porter CD. Retroviral hybrid LTR vector strategy: functional analysis of LTR elements and generation of endothelial cell specificity. *Gene Ther* 2004;**11**:775–83.
73. Greenberger S, Shaish A, Varda-Bloom N, et al. Transcription-controlled gene therapy against tumor angiogenesis. *J Clin Invest* 2004;**113**:1017–24.
74. Mavria G, Harrington KJ, Marshall CJ, Porter CD. In vivo efficacy of HSV-TK transcriptionally targeted to the tumor vasculature is augmented by combination with cytotoxic chemotherapy. *J Gene Med* 2005;**7**:263–75.
75. Di Pasquale G, Davidson BL, Stein CS, et al. Identification of PDGFR as a receptor for AAV-5 transduction. *Nat Med* 2003;**9**:1306–12.
76. Davidoff AM, Nathwani AC, Spurbeck WW, Ng YC, Zhou J, Vanin EF. RAAV-mediated long-term liver-generated expression of an angiogenesis inhibitor can restrict renal tumor growth in mice. *Cancer Res* 2002;**62**:3077–83.
77. Song W, Sun Q, Dong Z, Spencer DM, Nunez G, Nor JE. Antiangiogenic gene therapy: disruption of neovascular networks mediated by inducible caspase-9 delivered with a transcriptionally targeted adenoviral vector. *Gene Ther* 2005;**12**:320–9.
78. Condorelli G, Aycock JK, Frati G, Napoli C. Mutated p21/WAF/CIP transgene overexpression reduces smooth muscle cell proliferation, macrophage deposition, oxidative-sensitive mechanisms and restenosis in hypercholesterolemic apolipoprotein-E knockout mice. *FASEB J* 2001;**15**:2162–70.
79. Hedley SJ, Auf der Maur A, Hohn S, et al. An adenoviral vector with a chimeric fiber incorporating stabilized single chain antibody achieves targeted gene delivery. *Gene Ther* 2006;**13**:88–94.
80. Tang Y, Borgstrom P, Maynard J, et al. Mapping of angiogenic markers for targeting of vectors to tumor vascular endothelial cells. *Cancer Gene Ther* 2007;**14**:346–53.
81. Nicklin SA, Buening H, Dishart KL, et al. Efficient and selective AAV2-mediated gene transfer directed to human vascular endothelial cells. *Mol Ther* 2001;**4**:174–81.
82. White SJ, Nicklin SA, Buning H, et al. Targeted gene delivery to vascular tissue in vivo by tropism-modified adeno-associated virus vectors. *Circulation* 2004;**109**:513–9.
83. Stachler MD, Bartlett JS. Mosaic vectors comprised of modified AAV1 capsid proteins for efficient vector purification and targeting to vascular endothelial cells. *Gene Ther* 2006;**13**:926–31.
84. Baker AH, Sica V, Work LM, et al. Brain protection using autologous bone marrow cell, metalloproteinase inhibitors, and metabolic treatment in cerebral ischemia. *Proc Natl Acad Sci USA* 2007;**104**:3597–602.
85. Muller OJ, Kaul F, Weitzman MD, et al. Random peptide libraries displayed on adeno-associated virus to select for targeted gene therapy vectors. *Nat Biotechnol* 2003;**21**:1040–6.
86. van Beusechem VW, van den Doel PB, Gerritsen WR. Conditionally replicative adenovirus expressing degredation-resistant p53 for enhanced oncolysis of human cancer cells overexpressing muring double minute 2. *Mol Cancer Ther* 2005;**4**:1013–8.
87. Miller WH, Brosnan MJ, Graham D, et al. Targeting endothelial cells with adenovirus expressing nitric oxide synthase prevents elevation of blood pressure in stroke-prone spontaneously hypertensive rats. *Mol Ther* 2005;**12**:321–7.
88. Hallak LK, Merchan JR, Storgrad CM, Loftus JC, Russell SJ. Targeted measles virus vector displaying echistatin infects endothelial cells via alpha(v)beta3 and leads to tumor regression. *Cancer Res* 2005;**65**:5292–300.

89. Napoli C, Williams-Ignarro S, de Nigris F, et al. Beneficial effects of concurrent autologous bone marrow cell therapy and metabolic intervention in ischemia-induced angiogenesis in the mouse hindlimb. *Proc Natl Acad Sci USA* 2005;**102**:17202–6.
90. Napoli C, Loscalzo J. Niric oxide and other novel therapies for pulmonary hypertension. *J Cardiov Pharmcol Ther* 2004;**9**:1–8.
91. de Nigris F, Lerman LO, Napoli C. New insight in the transcriptional activity and coregulator molecules in the arterial wall. *Int J Cardiol* 2002;**86**:153–68.