

# Homing markers for atherosclerosis: applications for drug delivery, gene delivery and vascular imaging

Parul Houston\*, Jo Goodman, Alan Lewis, Callum J. Campbell, Martin Braddock<sup>1</sup>

*Cardiovascular Systems Unit, GlaxoWellcome Medicines Research Centre, Gunnels Wood Road, Stevenage, Hertfordshire SG1 2NY, UK*

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**Abstract** Endothelial dysfunction plays a major role in the pathogenesis of atherosclerosis. Pro-inflammatory cytokines such as interleukin-1 $\beta$  and tumour necrosis factor  $\alpha$  activate endothelial cells changing their resting phenotype to become pro-adhesive, pro-thrombotic and pro-atherogenic. Phage display in vivo biopanning has been used to identify peptide sequences that home to diseased regions of the vessel wall in low density lipoprotein receptor (LDLr) knockout mice. In LDLr knockout mice, peptide sequence determinants exhibiting organ specificity have been isolated. These sequences have applications for gene delivery, drug delivery and for improving contrast agents for vascular imaging. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Atherosclerosis; Homing markers; Gene delivery; Drug delivery; Vascular imaging

## 1. Introduction

Atherosclerosis is a focal disease of large- and medium-sized arteries and results in the formation of discrete raised lesions referred to as atherosclerotic plaques which occlude the vessel lumen. When fully advanced, these plaques restrict the flow of blood through the vessel and this often results in tissue ischaemia. Within the arterial wall, the intima is composed mainly of a single endothelial cell monolayer. The endothelium provides a sensory interface between the bloodstream and the vessel wall and is often described as the gatekeeper of vascular function [1]. Initiation of lesion formation is brought about as a result of various forms of insult to the arterial endothelium which include pro-inflammatory cytokines and risk factors such as hypercholesterolaemia, smoking, diabetes and hypertension [2]. This endothelial injury results in the endothelium developing a pro-adhesive, pro-atherogenic, pro-thrombotic phenotype which manifests itself as increased inflammatory leukocyte adhesion, promotion of smooth muscle cell migration and proliferation, modulation of extracellular matrix composition, modulation of vas-

cular tone and susceptibility to thrombus formation [3]. Animal models of atherosclerosis include the apoE and low density lipoprotein receptor (LDLr) knockout mouse models which, when maintained on a high fat 'Western diet', mimic aspects of the human disease. Animals exhibit elevated levels of circulating pro-inflammatory cytokines, enhanced endothelial expression of adhesion molecules at sites of lesion formation and display lesion formation [4–6].

The identification of targeting moieties that home specifically to sites of atherosclerotic plaque development has numerous potential therapeutic applications. These include targeting gene, or small molecule, therapeutic agents for modifying vessel wall pathology and as directed agents for improving intravascular imaging [7]. Such targeting agents may also identify novel disease-specific ligands that may lead to the identification of novel receptors and potential new drug targets. In vivo biopanning was developed to identify peptide ligands, derived from phage display libraries, that were capable of homing to specific organs in a whole animal [8]. This pioneering work was extended to identify a number of tri-peptide motifs that homed to the vasculature of a range of different organs such as the lung, spleen, skin and pancreas [9]. Peptides capable of homing to angiogenic vessels of human breast carcinoma xenografts were later identified [10], and one of the motifs isolated was the integrin binding RGD motif. A further motif, the NGR sequence, was found to be a cell adhesion molecule-specific motif. These peptides were conjugated to the cytotoxic cancer therapeutic agent doxorubicin and when injected into mice bearing breast carcinomas, this agent resulted in inhibition of tumour growth and metastasis and improved mouse survival for up to 6 months. This study was the first example where in vivo biopanning was used in a disease model of clinical relevance. Further studies have identified peptides that mediate anti-tumour activity. A peptide capable of inhibiting matrix metalloproteinase 9 activity by phage panning in vitro was identified with tumour growth and invasion being prevented when the isolated peptide was injected into tumour bearing mice [11]. Tumour homing peptides have also been successfully used to direct pro-apoptotic peptides to angiogenic vessels in mouse tumours [12].

We have used phage display in vivo biopanning to identify peptide ligands that home specifically to sites of diseased vessel wall in the LDLr knockout mouse model of human atherosclerosis. Further characterisation of one such peptide was performed using an in situ binding protocol to frozen tissue sections. Binding of this phage to diseased vessels was inhibited by co-injection of phage with unconjugated peptide.

\*Corresponding author. Fax: (44)-1438-764488.  
E-mail: pb6174@glaxowellcome.co.uk

<sup>1</sup> Present address: Disease Sciences Section, Discovery Biosciences Division, AstraZeneca R&D Charnwood, Bakewell Road, Loughborough, Leicestershire LE11 5RH, UK.

## 2. Materials and methods

### 2.1. Phage display libraries

Libraries of random peptides expressed at the N-terminus of the fd phage major coat protein pVIII, were created as described [13]. Each library was constructed as a fusion between the wild type (wt) pVIII major coat protein followed by a (Gly<sub>4</sub>Ser)<sub>3</sub> spacer arm. Library ON2604 of structure GGC(X)<sub>12</sub>C(G<sub>4</sub>S)<sub>3</sub> was a constrained 12-mer library (where X is any amino acid and (G<sub>4</sub>S)<sub>3</sub> represents a glycine: serine spacer arm) and was obtained as a gift from Affymax (Palo Alto, CA, USA).

### 2.2. Determination of phage titre

Phage were titred in *Escherichia coli* strain AR1392 (Affymax, Palo Alto, CA, USA) according to [14]. Dilutions of the phage to be titred were prepared in PBS. Typically dilutions between 10<sup>-2</sup> and 10<sup>-8</sup> were prepared. 10 µl of the diluted phage was added to 100 µl of growing cells and then incubated at 37°C for 20 min without shaking. The infected cells were then plated out on L-amp (100 µg/ml) plates and incubated overnight at 37°C.

### 2.3. In vivo biopanning of phage display libraries in the LDLr knockout mice

LDLr knockout mice were maintained for 24 weeks on a Western diet to induce atherosclerotic lesions [6]. Mice were anaesthetised with Avertin (0.017 ml/g). 10<sup>10</sup> tu of the phage display library in a total volume of 500 µl DMEM (Sigma, Poole, Dorset, UK) was injected into the mouse tail vein. 4 min after phage administration, the mouse was perfused through the heart with 5–10 ml of DMEM. Regions of vasculature that displayed lesions, namely the subclavian artery, the carotid arteries and the aorta were dissected out, weighed and placed in DMEM containing a cocktail of protease inhibitors (PI) used as recommended by the manufacturers (Boehringer Mannheim). The vessel segments were homogenised using a Dounce homogeniser and the tissue homogenate was washed three times in ice-cold wash buffer (DMEM-PI/1% BSA). 10 µl of the homogenate was then titred in triplicate, and the phage rescued from the tissue by *E. coli* infection and phage amplification. A total of 250 individual colonies were selected from round 1 of the biopanning procedure and amplified in *E. coli*. For the second round of biopanning, 10<sup>9</sup> tu of purified phage was then re-injected into a second LDLr knock-out mouse. The whole panning and amplification process was repeated twice more. 100 clones from rounds 2 and 3 were then selected for sequencing. Peptides or peptide motifs that appeared consistently in rounds 2 and 3 of the panning process were retained for DNA sequencing and bioinformatics analysis.

### 2.4. DNA sequencing and bioinformatics

Samples were sequenced using the automated ABI sequencing facilities at GlaxoWellcome using primers to the p8V5 phagemid, sequence, 5'-TGAGGCTTGACGGGAGTC-3'. Peptide sequences were aligned using the GCG (Wisconsin) software package. Peptide identities were compared using an analysis script (A. Lewis, unpublished).

### 2.5. Organ-specific homing of individual clones

Specificity of phage homing to key organs in vivo was determined by individual phage recoveries from tissue homogenates and by testing for selective inhibition of homing by the unconjugated peptide. Peptides were synthesised with an N-terminal acetylation and were HPLC purified (Sigma Genosys) and reconstituted in sterile distilled water. Free peptide (1 mg) was co-injected with 10<sup>10</sup> tu of the specific phage clone. Test organs and regions of diseased vasculature were isolated, homogenised and the number of phage recovered per gram of tissue determined by amplification in *E. coli* as described above. All phage titrating experiments were carried out in triplicate. A control, insertless phage clone was injected into mice in order to determine background levels of phage binding.

### 2.6. In vitro binding of homogeneous phage clones to frozen tissue sections

Frozen tissue sections of diseased mouse vasculature were cut at 7 µm and fixed in ice-cold acetone. After rehydration in PBS, the sections were incubated in PBS/0.03% H<sub>2</sub>O<sub>2</sub> for 20 min at room temperature (RT), followed by a 1 h block in PBS/2% BSA. Homogeneous phage preparations at concentrations of 10<sup>9</sup>, 10<sup>8</sup> and 10<sup>7</sup> tu

per section were applied for 2 h at RT. Following three washes with PBS/0.1% Triton X-100 and PBS, rabbit anti-fd phage antibody (Sigma) was applied to the sections for 1 h at RT at 1:1000 dilution. After a further three washes with PBS/0.1% Triton X-100 and PBS, biotinylated anti-rabbit IgG antibody was applied to the sections at 1:200 dilution. PECAM-1 staining, to show endothelial cell integrity, was performed using an antibody directed against PECAM-1 (R&D systems) at 1:100 dilution. Antibody detection was then carried out using the Vectastain Elite kit (Vector Laboratories). In all experiments, a minus-phage and a minus-anti-fd antibody control section was incorporated.

## 3. Results

### 3.1. Sequential enrichment of phage homing to atherosclerotic vasculature in the LDLr knockout mouse

To demonstrate that we could enrich for a heterogeneous population of phage capable of homing to diseased vessel wall, sequential rounds of biopanning were performed in the LDLr knockout mouse (Fig. 1). 10<sup>10</sup> of the constrained library phage ON2604, were injected into the tail vein of an LDLr knockout mouse previously maintained on a Western diet. Following circulation of the phage, vascular perfusion with DMEM and animal sacrifice, diseased areas of vasculature were removed. The number of phages recovered per gram of tissue was determined. The phage eluates were amplified and the biopanning process was repeated a further two times yielding an increase in phage recovery from diseased vasculature of approximately 1000 times (Fig. 1). 100 clones were selected for sequence analysis. Ten clones, sequences of which are shown in Table 1, were isolated repeatedly in rounds 2 and 3 of panning. One clone (2.6) of sequence CLVEAYPGL\*VRSC (where \* denotes a stop codon) was picked for further characterisation.

### 3.2. Binding specificity of phage clone 2.6 to atherosclerotic tissue

Phage clone 2.6 was tested for binding specificity to diseased vasculature from the LDLr knockout mouse (Fig. 2). Regions of vasculature displaying advanced atherosclerotic lesions such as the aorta were isolated from an LDLr knock-

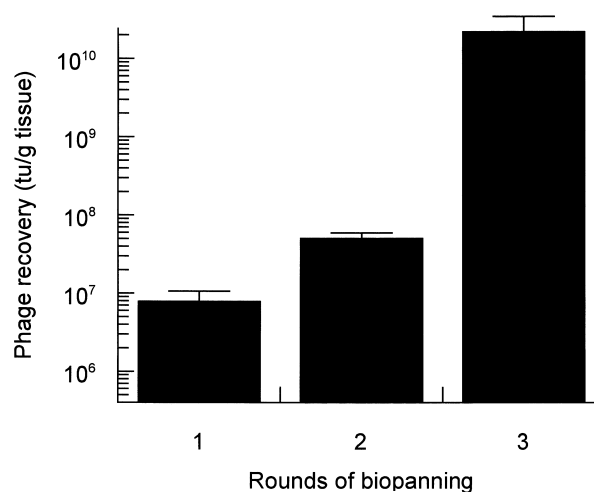


Fig. 1. Sequential enrichment of phage homing to atherosclerotic vasculature in the LDLr knockout mouse. 10<sup>10</sup> tu of constrained 12-mer phage library ON2604 was sequentially panned for three rounds. Phage recovery was determined as tu phage per gram of tissue.

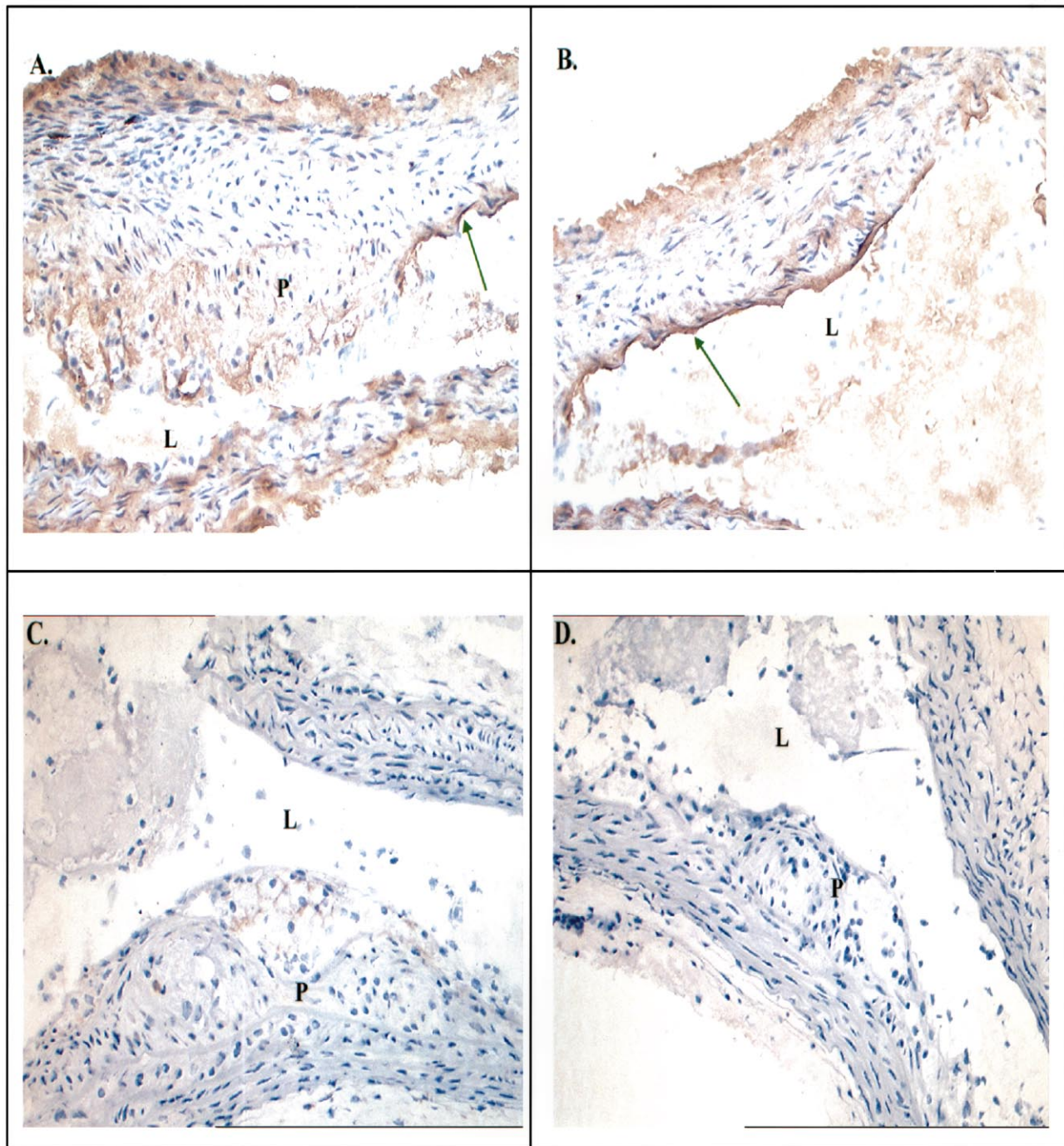


Fig. 2. Selective binding of phage clone 2.6 to regions of atherosclerotic plaque development.  $10^8$  tu of clone 2.6 or a control insertless phage was added to frozen tissue sections of vasculature from an LDLr knockout mouse and binding detected immunohistochemically using antibodies to the phage coat protein. Scale bar represents 10  $\mu$ m. A: Binding is within the plaque (P) and on the endothelium (green arrow). L = lumen. B: Endothelial staining in a region of vessel adjacent to plaque development (green arrow). L = lumen. C: Insertless phage binding to atherosclerotic vessel. P = plaque, L = lumen. D: Staining in atherosclerotic vessel in the absence of secondary biotinylated anti-rabbit IgG antibody. L = lumen.

out mouse. Frozen sections of tissue (7  $\mu$ m) were incubated with  $10^8$  tu of undiluted phage clone 2.6 to allow binding. In addition, phage binding was performed at a range of concentrations using serial dilutions of phage at  $10^{10}$ ,  $10^9$ ,  $10^8$  and  $10^7$  tu per section. Results are presented for tissue sections incubated with  $10^8$  tu phage per section. Phage binding was determined immunohistochemically. Phage binding was local-

ised predominantly within the plaque and on the endothelium, with lesser binding detectable in the media and the adventitia (Fig. 2A). Intense endothelial cell staining was detected in a diseased section of aorta in the absence of a neointimal lesion (Fig. 2B). Sections of diseased vessel probed with a homogeneous phage preparation lacking a peptide insert, or clone 2.6 without secondary antibody functioned as negative controls

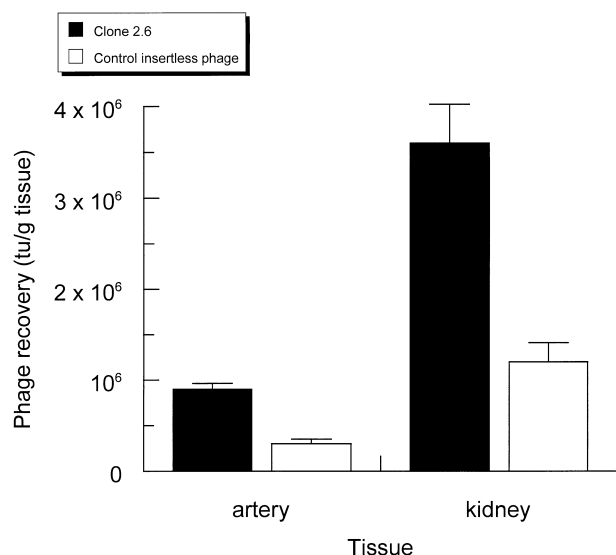


Fig. 3. Differential binding of phage clone 2.6 to artery and kidney from LDLr knockout mouse.  $10^{10}$  tu of phage were injected into a mouse and phage recovered from kidney and arteries. Recovery is expressed as tu phage per gram tissue.

(Fig. 2C,D). There was no detectable binding of phage clone 2.6 to the vasculature of a non-diseased mouse of the same C57 black parental strain. The presence of an intact endothelial cell layer in both the diseased and control tissue sections was determined to be positive by immunohistochemistry using PECAM-1 antibody (data not shown).

### 3.3. Differential binding of phage clone 2.6 to artery and kidney from LDLr knockout mouse

Injection of  $10^{10}$  tu of phage clone 2.6 into the LDLr knockout mouse resulted in significant phage recovery from both isolated diseased vasculature and the kidney when compared to the recovery of a control non-specific insertless phage clone (Fig. 3). A high level of non-specific phage binding to other organs isolated in this study, such as liver, spleen and lung was observed (data not shown, [9]).

### 3.4. Competition for phage homing in vivo by unconjugated peptides

In order to determine whether there was in vivo specificity for this phage to areas of diseased vasculature, a selection and competition biopanning experiment was performed. Two peptide preparations were used, a linear 9-mer peptide

Table 1  
Peptide sequences identified following in vivo panning in the LDLr knockout mouse

Clone	Sequence
2.6	CLVEAYPGL*VRSC
2.20	CRM*KARRTPVNSC
3.5	CVR*SETCERYKTC
3.8	CRERVSPLKSM*WC
3.18	CRSRRV*SWVHSLC
3.44	C*LRCRMTMPVRR
3.46	CL*RFCRVRGTFTC
3.70	CMGYGW*PPVRDGC
3.79	CHANPE*RCMTAWC
3.97	CLASALWNYES*AC

\*Denotes a stop codon within the peptide sequence.

(CLVEAYPGL) designated peptide 1 and a constrained 14-mer peptide, where a serine residue had been inserted into the site of the stop codon (CLVEAYPGLSVRSC) and was designated peptide 2. Co-injection of  $10^{10}$  tu of the phage clone 2.6 with each of the free peptides resulted in a 26% binding inhibition of phage to the vasculature by peptide 2. No significant inhibition of binding was demonstrated by the linear 9-mer peptide (Fig. 4).

## 4. Discussion

In this paper we demonstrate the application of in vivo biopanning to a murine model of disease, the LDLr knockout mouse model of atherosclerosis. The expression of endothelial markers ICAM-1, VCAM-1, E-selectin and PECAM-1 has been extensively studied in both the LDLr and the apoE knockout mouse models and shown to be upregulated at sites of lesion development [5,15]. These observations, taken together with previous studies [9] suggest that the vascular endothelium displays a heterogeneous range of markers that may function as molecular addresses. By sequential panning of a constrained, cyclic 12-mer phage library, a phage population enriched 1000 fold was obtained after three rounds of biopanning. A previous study reported an enrichment ratio of 100 over three rounds of biopanning for phage that homed to lung, kidney and brain [9]. Our data suggests that a highly significant enrichment of phage specific to the diseased vasculature was evident between each round of panning in the LDLr knockout mouse.

Sequence analysis of 100 clones picked from rounds 2 and 3 of panning revealed 10 peptide motifs that appeared repeatedly in each round, of which one motif, LVEA was the most common. The full 12 amino acid sequence incorporated a stop codon at position 9, suggesting that a truncated fusion product may have been created. A stop codon does not necessarily mean that a truncated product has been created since there are examples where translational readthrough may occur from ORF within phage display libraries. This was demonstrated

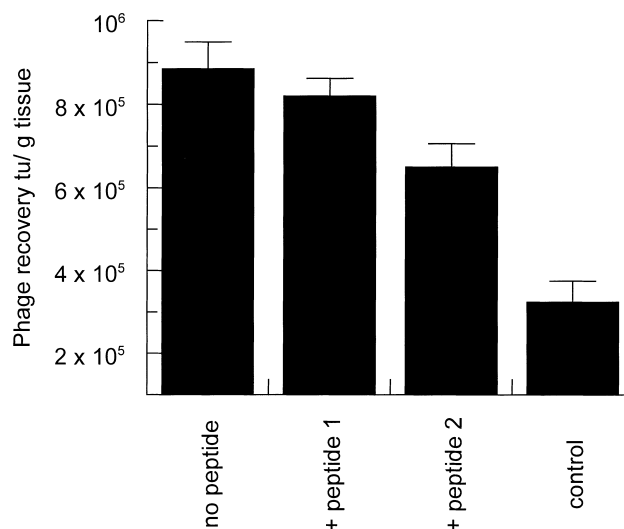


Fig. 4. Competition for phage homing in vivo by unconjugated peptides.  $10^{10}$  tu of phage clone 2.6 was injected into an LDLr knockout mouse with a 9-mer (peptide 1) or 12-mer peptide (peptide 2). An insertless phage preparation at  $10^{10}$  tu per animal was used to determine background binding.

by a translational recording mechanism whereby ribosomes switched reading frames or read past stop codons when encountering a signal in the nucleotide sequence of the mRNA [16]. Isolation of peptide sequences which include stop codons is a common occurrence when using this technique since they often represent faster growing phage clones. Nevertheless, such truncated displayed sequences have shown potential in homing experiments targeted at specific tissues (R. Pasqualini, personal communication). Clone 2.6 showed specific homing to regions of the vascular endothelium of diseased vessels and within the atherosclerotic plaque. This is the first time that this has been achieved *in vivo*. Biopanning with clone 2.6 resulted in significant phage recovery from both isolated diseased vasculature and the kidney, when compared to the recovery of a control non-specific insertless phage clone. A high level of non-specific phage binding to other organs isolated in this study, such as liver, spleen and lung was observed (data not shown). Non-specific phage homing to liver, spleen and lung tissue has previously been observed [8]. This would be expected in tissues such as the liver and spleen since these organs form part of the reticulo-endothelial system and represent 'sinks' for non-specific phage binding.

The enhanced recovery of clone 2.6 from the kidney may be due to the observation that atherosclerosis in the renal arteries is very common in man [17] and has also been demonstrated in murine models of atherosclerosis [4]. However it may well be possible that some non-specific phage accumulation may have occurred due to renal mesangial cell phagocytosis.

Competition for phage binding, as determined by phage recovery was greater with the 12-mer as opposed to the 9-mer unconjugated peptide which may suggest the phage particle was more likely to have produced a fully constrained 12-mer fusion product whereby readthrough of the stop codon had occurred.

The LVEA motif displayed sequence homology to VCAM-1, eNOS, TNF- $\alpha$  receptor and VEGF receptor. The role of each of these molecules in the pathogenesis of atherosclerosis is well documented [2]. Whilst it is recognised that clone 2.6 could be represented in these proteins, one cannot formally rule out the possibility that this motif may well form part of a novel cell surface receptor.

Further studies in our laboratory will be aimed at addressing the exploitation of this peptide as an agent for targeting both drug and gene delivery to sites of atherosclerosis. Conjugation of targeting peptide moieties to adenoviral, retroviral and liposome vectors presents enormous potential for improving future gene therapy technology [18,19]. It may also be

feasible to the couple peptide targeting together with a transcriptional targeting strategy such as promoters that respond to laminar shear stress [20]. In this manner distinct foci of wounded endothelium may be targeted for gene therapy. Finally, an interesting extension of the application of targeted liposomes may be provided by their utility in improving imaging agents for atherosclerosis [21].

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