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Targeting of peptides to restenotic vascular smooth muscle cells using phage display in vitro and in vivo

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Abstract

Restenosis after angioplasty occurs in 30–40% of the treated patients. To develop a strategy to deliver drugs to restenotic lesions, we selected phages that bind to proliferating vascular smooth muscle cells (VSMC), from a random constraint 15-mer peptide phage display library. Phages were selected for binding to cultured primary aortic VSMC (in vitro biopanning) and selected for binding to denudated carotid arteries in mice (in vivo biopanning). In vitro biopanning did not result in a consensus sequence, but recurring FLGW and LASR amino acid motifs were identified. In vivo biopanning resulted in two consensus peptides 5G6 (CNIWGVVLSWIGVFPEC) and 5E5 (CESLWGGLMWTIGLSDC). Surprisingly, these two sequences were recovered after both in vitro and in vivo biopanning, but predominantly in vivo. Moreover, a strong recurring motif, IGR, was identified in the in vivo clones. The consensus phages 5G6 and 5E5 bind selectively to VSMC compared to other cell types. Furthermore, they bind preferentially to proliferating VSMC compared to VSMC that were growth arrested, and are effectively internalized by their target cells. The specific binding capacities of 5G6 and 5E5 phages suggest that these peptide sequences can be used for targeting of restenotic lesions, in which proliferating VSMC are the dominant cell type.

Keywords: Vascular smooth muscle cell; Restenosis; Phage display; Cell targeting

1. Introduction

Restenosis is an inflammatory process similar to atherosclerosis, in which the migration and proliferation of vascular smooth muscle cells (VSMC) plays an important role [1]. These events are associated with a phenotypic change of VSMC, characterized by modification in gene expression [2]. Promising therapeutic effects in a rat model can be accomplished by cytostatic drugs such as taxol [3], or adenoviral gene therapy [4]. However, a major complication of this approach is delivering sufficient amounts of these therapeutics to the restenotic plaque, as systemic administration of high doses of cytostatic drugs or adenovirus can lead to cytotoxic effects [5].

Local delivery of therapeutics to prevent restenosis are invasive and most of them limited to the moment of angioplasty itself. To apply the drug of interest, a coated or porous balloon [6,7] can be used during angioplasty. Other local delivery systems involve catheter-based application [8,9] or direct pericardial injection [10]. However, not all of the diseased vessels will be readily available for local injection. Systemic treatment, in which the delivery system after intravenous administration will be able to specifically target the restenotic plaque, could overcome these problems and also enable to continue the treatment after angioplasty.

Systemic applications in restenosis require a ligand that selectively targets the proliferating VSMC in the restenotic plaque, thereby greatly enhancing its efficacy.

Antibodies specific for proliferating VSMC have been shown useful in targeting atherosclerotic lesions in humans and rabbits upon systemic injection. Moreover, modification of the antibody with poly-L-lysin for additional negative charge was necessary to reduce a non-specific uptake [11,12]. Whereas atherosclerotic lesions in the rabbit are only a few cell layers thick, the size of antibodies could

Abbreviations: VSMC, vascular smooth muscle cells; PBS, phosphate buffered saline; NCS, newborn calf serum; BSA, bovine serum albumin; WT, wild type

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cause a problem in penetration of restenotic plaques, which are characterized by a dense extracellular matrix. Other limitations to this approach may involve the high costs of antibody production, toxicity and the immunogenicity of their targets [13,14].

As an alternative for antibodies, peptides with high cell binding capacity and specificity may be used. Synthetic peptides with IC_{50} values within the micromolar range [15,16] and even nanomolar range when binding to structural proteins like glycosaminoglycans and integrins [17–19] have been reported. Furthermore, peptides are small molecules that are easy to synthesize and are easily coupled to the desired drug. Using a peptide phage display library, large numbers of peptides can be screened for binding to the target cells in vitro using biopanning [20–22]. Major advantage of this approach is that no prior knowledge of both ligand and receptor involved in the interaction is required.

Phage display has also been shown to be very useful in vivo, as phages are even stable enough to be injected systemically and can readily be isolated from tissues without loss of infectivity. Moreover, in vivo selection of phages has resulted in peptides that are very effective and specific in targeting the organs they were selected for upon systemic injection [23–25]. For this reason, we selected peptides that bind to mouse proliferating VSMC both in vitro in primary cultured VSMC as well as in vivo in a denudated mouse carotid artery using a random constraint 15-mer peptide phage display library. The selected phages selectively bind to VSMC compared to other cell types, with a preference for proliferating VSMC.

2. Materials and methods

2.1. Cell culture

VSMC: aortas were isolated from male C57Bl6 mice, washed with sterile phosphate buffered saline (PBS) and digested in Hanks containing 2 mM L-glutamin, 0.3% bovine serum albumin (BSA) and 1 mg/ml collagenase for 90 min at 37 °C. Resulting cells were pelleted and washed with sterile PBS. The cells were plated onto 0.1% gelatin-coated plates using DMEM (Gibco) + 10% newborn calf serum (NCS). The cells were identified as VSMC by positive and homogeneous immunocytochemical staining for α-SM-actin and VCAM-1 (>90%), known VSMC markers. Also, the cultured cells were positive in RT-PCR for α-SM-actin, SM-22α, vimentin and osteopontin (Fig. 1). The cultured cells were used at high passage numbers (passage 13– 30) to ensure a proliferative, dedifferentiated character. The morphology of the cells resembled the so-called 'cobblestone'-like phenotype of dedifferentiated aortic SMC at confluency. To obtain the proliferating phenotype, VSMC were seeded at low density the day before use, whereas differentiated VSMC were allowed to differentiate for 3 days in the presence of 250 U/ml heparin or 1.5% NCS at high cell numbers.

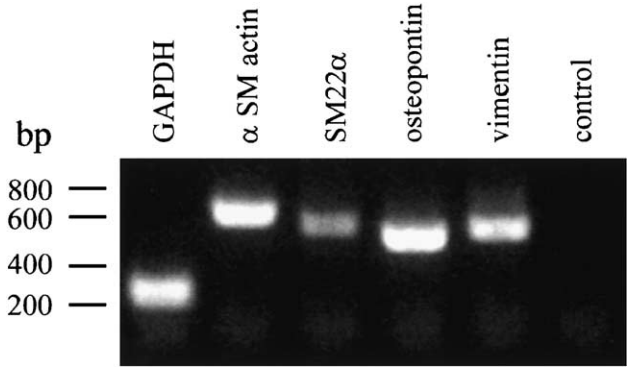

Fig. 1. RT-PCR analysis of VSMC cDNA with GAPDH, α-SM-actin, SM22α, osteopontin and vimentin primers shows the presence of these VSMC marker RNAs.

2.2. Denudation of mouse carotid artery

Endothelial denudation of the arteria carotis communis was induced by introducing a guidewire essentially as described by De Geest et al. [28]. Female ApoE$^{-/-}$ mice were anesthetized with a mixture of 15 mg/ml ketamine, 5.7% Thalomonal, and 1% Hypnorm in PBS, 75–100 μl/10 g body weight, subcutaneously. Four ligatures were placed around the arteria carotis communis, arteria carotis externa and interna. Through a small incision distal from the branch of the arteria carotis externa, a rough wire of 0.45 mm in diameter (Cook) was introduced via arteria carotis externa into the arteria communis. Denudation was induced by triple withdrawal of the wire.

2.3. Biopanning procedures

2.3.1. Phage display library

A random 15-mer constrained peptide library was constructed in the pComb8 phagemid, and consists of a random 15 amino acid sequence, flanked by two cysteine residues for a cyclic conformation. The library contains 1.8×10^8 peptide variants that are expressed at the N-terminus of the major coat protein pVIII [26].

2.3.2. In vitro biopanning procedures

VSMC were plated into 60-mm dishes, at 7×10^5 cells/dish, 20 h before selection. The cells were washed and incubated in Hanks-complete (Hanks containing 2 mM L-glutamin and 1% BSA) for 2 h at 37 °C. One hundred library equivalents, 2×10^{10} cfu, in 1 ml Hanks-complete + 100 μM chloroquine, were added and incubated at 37 °C for 1 h. After extensive washing with cold Hanks-complete, all phages that associate with the VSMC but were not internalized, were removed using either 0.2 M glycine/HCl, pH 2.2 for 15 min on ice. Cells were lysed in 1 ml cold NP-40 lysis buffer (50 mM Tris pH 7.5, 0.5 mM EDTA, 75

mM NaCl, 0.5% NP-40, 1 mM PMSF, 20 μg/ml aprotinin, 1 μg/ml leupeptin) for 1 h on ice to release internalized phages. The cell debris was scraped from the plate using a cell scraper, and vortexed briefly. The selected phages were amplified and titered using XL1-Blue *E. coli* bacteria and VCS-M13 helper phage (Stratagene) and subsequently sequenced as described [26,27].

2.3.3. In vivo selection procedure

Phages (10^{10} cfu) that were selected in vitro for overall binding to VSMC during three rounds were injected in the tail vein of denudated mice, 7 days after denudation. After 2 h, the mice were killed by an overdose of anesthesia and perfused with Krebs–Ringer solution for 10 min. The treated arteries were removed and dissected mechanically first, followed by enzymatic digestion of the arteries in Hanks + 2 mM L-glutamin + 0.3% BSA and 1 mg/ml collagenase, 1 mg/ml trypsin inhibitor and 0.47 U/ml elastase I for 1 h at 37 °C. The reaction was terminated by adding 1 mM PMSF. The detached cells were collected by centrifugation, dissolved in NP-40 lysis buffer and lysed at 4 °C for 1 h to collect phages.

2.4. Phage binding assay

Cells were plated into 96-well plates (1×10^4 c/w) the day before use. The cells were washed and incubated in Hanks-complete at 37 °C for 2 h. Phages were added and incubated with the cells for 2 h at 37 °C. Non-bound phages were washed away extensively with Hanks-complete. The cells and phages were fixed using ice-cold methanol, 10 min at −20 °C. After fixation, the cells were washed with PBSS (PBS + 0.1% saponin) + 5% milk and incubated with biotinylated polyclonal anti-M13 serum (5 Prime → 3 Prime). After this incubation, the cells were washed and incubated with PO-labeled streptavidin (Amersham Life Science). Peroxidase activity was detected with TMB (0.3 mg/ml + 0.03% H_2O_2). The reaction was terminated with 1 M H_2SO_4 and measured at 450 nm.

3. Results

3.1. In vitro selection

For biopanning, we used a random 15-mer constraint peptide library in combination with proliferating cultured aortic VSMC. Phages were added to a monolayer of dedifferentiated, proliferating VSMC. After 1 h incubation, non-binding phages were washed away. Phages bound to the outside of the VSMC were removed by an acid wash. Subsequently, cells were lysed to release internalized phages. Collected phages were amplified and used for four subsequent rounds of biopanning. Sequence analysis of a total of 45 randomly picked clones from two separate selections revealed no evident consensus sequence. However, some recurring motifs, mainly FLGW and LASR, were identified (Table 1). Next, 96 clones of two separate selections were picked and grown individually in a 96-well plate. The clones were tested for binding to VSMC in a phage binding assay and clearly show a high binding capacity for VSMC compared to binding of an unselected phage population and wild-type phages (Fig. 2).

3.2. In vivo selections

After five rounds of biopanning, still a wide variation in sequences was detected. To find a consensus, phages were subjected to in vivo selection upon injection in denudated mice. For this purpose, endothelial cells aligning the carotid arteries of ApoE$^{-/-}$ mice were removed using a rough guidewire [28], leaving the VSMC exposed (Fig. 3). First,

Table 1
Motif analysis of resulting clones. Homologies between amino acid residues are shaded

possible motif											**L**	**A**	**S**	**R**	**v** / **f**	**s** / **g**	**s**		**F**	**L**	**G**	**W**	**e** / **w**			
A. 5x in vitro											L	A	S	R	V	S	S		F	L	G	W	G	S	N	L
							E	S	F		W	A	S	R	F	S	S			L	G	G	M	A		
											S	A	S	F	F	S	W		L	F	G	W	E	L	A	
					S	D	M	W			L		S	R	L	L	F		T	F	L	G	W			
					V	L	V	E			L	A	S	L	V	G	L		S	L		W	E			
											L	A	N	G	W	G	C		F	L	G	C	W	F	H	G
							H	V			L	A	M	V	V	G			F	L	G	V	E	W	F	
possible motif												**I**	**G**	**R**												
B. 3x in vitro	N	I	W	G	V	V	L	S	W		I	G	V	F	P	E										
+ 3x in vivo	E	S	L	W	G	G	L	M	W	T	I	G	L	S	D											
		S	F	L	T	R	L	G	C	E	I	G	R	F	L	R										
						D	C	T	V	R	I	G	R	V	C	M	A	A	S							
					N	G	V	I	F	S	W	G	R	G	W	E	L	A								

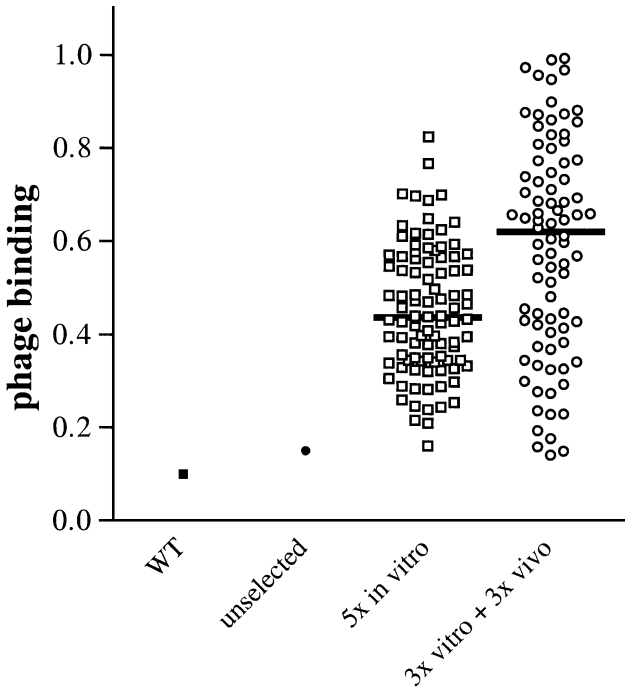

Fig. 2. Binding of randomly picked phage clones to proliferating VSMC after different selection procedures. The binding of wild-type (■) and unselected phages (●) to proliferating VSMC were compared to phages that were selected for internalization by proliferating VSMC in different selection procedures. Clones were picked at random after five rounds of in vitro biopanning (□, $n=96$) and after three selection rounds in vitro without removal of non-internalized phages, followed by three selection rounds in vivo (○, $n=84$). Binding was measured using a phage binding assay. Each point represents the average binding of one clone in two separate assays. Median values are indicated (black lines).

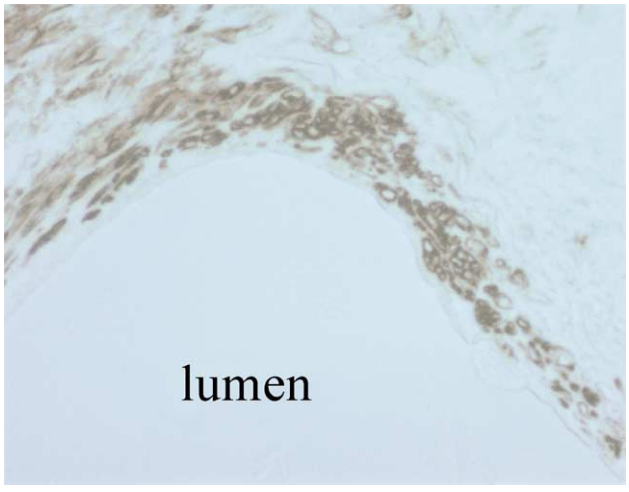

Fig. 3. Carotid artery of an ApoE$^{-/-}$ mouse at day 7 after denudation. Vascular smooth muscle cells were stained using an antibody against α-smooth muscle actin (clone 1A4), a marker for vascular smooth muscle cells (brown staining). Vascular smooth muscle cells are exposed at the lumenal site, surrounded by excreted extracellular matrix (unstained). Magnification ×400.

we determined at what day after denudation most VSMC were exposed and proliferating. To ensure the formation of a plaque, a wire with a diameter of 0.45 mm was used. This treatment caused a significant loss of cells at the luminal surface. It took about 5 days for the cells to recover, as shown by HE staining. The amount of proliferation was determined by BrdU-staining, showing that proliferation was maximal at day 7 after denudation (not shown). Before in vivo selections, phages were selected by three rounds of biopanning on cultured VSMC, as described. In this case, non-internalized phages were not removed, to ensure the largest possible variation within this pre-selection. After three rounds of in vitro biopanning, phages were injected into the tail vein of denudated mice, 7 days after treatment, and allowed to circulate for 2 h. After total body perfusion, the carotid artery was removed and binding phages were collected. After three rounds of additional in vivo biopanning, 84 clones were picked and grown individually in 96-well plates and tested for binding to VSMC in a phage binding assay (Fig. 2).

Fourteen phages with highest binding capacity were sequenced. This time, a consensus peptide sequence, CNIWGVVLSWIGVFPEC, was present which represented 75% of the binding population. Surprisingly, the consensus sequence was identical to phage 5G6, which was also selected after biopanning in vitro. Another phage, 5E5 (CESLWGGLMWTIGLSDC), which was recovered after binding to denudated carotid arteries, was also recovered after in vitro biopanning on VSMC. This sequence occurred only once in both selections (Table 2). Also, a clear motif, IGR, was present in all of the in vivo selected peptides (Table 1).

3.3. Specificity of selected phages for VSMC

To test the specificity of the selected phages to target restenotic VSMC, they were tested for binding to other cell types that will be encountered upon intravenous injection. For this reason, VSMC, murine fibroblasts (3T3), endothelial cells (H5V), murine monocytes and macrophages (RAW) and liver cells (AT3) were cultured in 96-well plates and the binding of phages to each cell type was determined. The background uptake of unselected phages and wild-type phage by RAW cells was relatively high, most likely due to phagocytosis. Although the binding to 3T3 cells was generally lower than the binding to VSMC, this difference was

Table 2
Occurrence of peptide sequences. Binding to VSMC was determined using phage binding assay

Clone	Amino acid sequence	Occurrence		Binding
		in vitro	in vivo	VSMC
5G6	CNIWGVVLSWIGVFPEC	1/45	10/14	++++
5E5	CESLWGGLMWTIGLSDC	1/45	1/14	++++
2G3	CSASFFSWLFGWELAC	1/45	0/14	++++

not significant. This can be explained by the fact that the phenotype of proliferating VSMC becomes more fibroblast-like during culturing, due to dedifferentiation. The consensus phage 5G6 shows significant specific binding to VSMC, compared to wild-type and unselected phages, as well as the other phage that was selected both in vitro and in vivo, 5E5. Another in vitro selected clone, 2G3, chosen for its high binding capacity, binds with significant specificity to VSMC as well (Fig. 4).

3.4. Specificity of selected phages for proliferating VSMC

Restenotic VSMC differ from normal VSMC. Restenotic VSMC are VSMC that have dedifferentiated into a more fibroblast-like stadium. They no longer contract, but are able to migrate and proliferate, and to synthesize large amounts of extracellular matrix, which leads to occlusion of the arterial lumen. To be successful in targeting restenotic lesions, selected phages must be able to bind to VSMC with a restenotic phenotype. This means that they have to be specific, mainly, for proliferating VSMC with a dedifferentiated phenotype.

Starvation at low serum is a common method to inhibit cell growth and synchronize cells. More specific is inhibition by heparin, which inhibits the growth of VSMC specifically [29]. We tested both methods. At indicated time points, cells were detached using trypsin and counted. As suspected, low serum conditions (1.5% NCS) were very sufficient in inhibiting the growth of VSMC, but VSMC cells did not survive serum-free conditions in the presence of 1% BSA. Significant inhibition of growth was also obtained at heparin concentrations as low as 5 U/ml. Total inhibition was accomplished at concentrations between 50 and 500 U/ml (Fig. 5A and B), while viability was unaffected.

The amount of differentiation was determined by Western blotting and staining for α-SM-actin, a differentiation marker for VSMC. α-SM-actin expression was induced 3 days after the addition of 50–500 U/ml heparin, whereas without heparin, only a minor α-SM-actin expression was obtained only after 6 days, but never reached the same levels of cells induced with heparin (Fig. 5C).

Both heparin-inhibited and serum-starved VSMC were compared to proliferating VSMC for binding of the selected

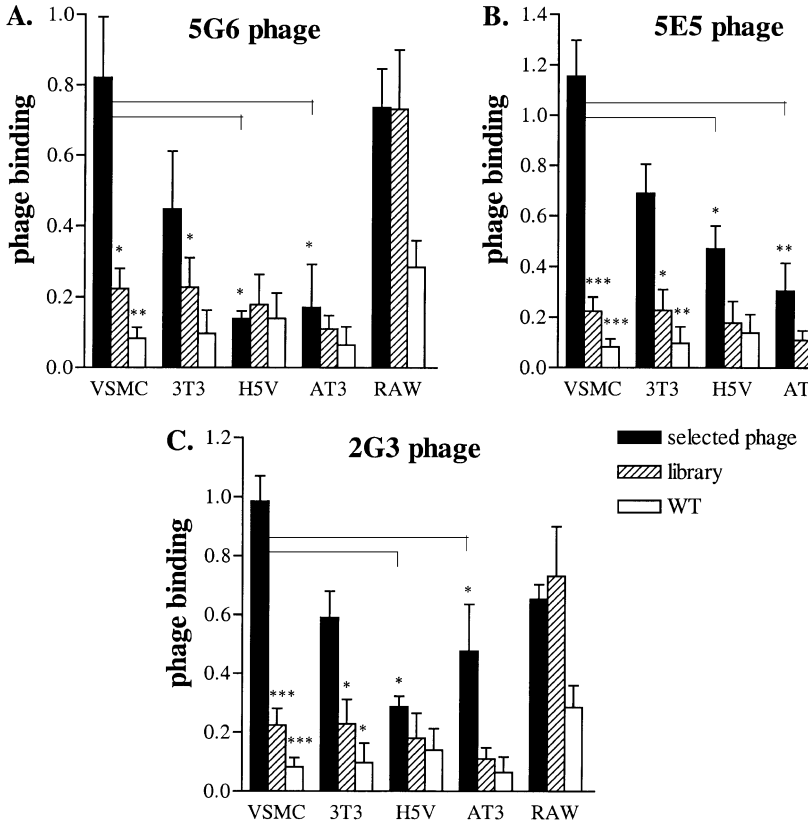

Fig. 4. Binding of selected clones to different cell types. Cells were seeded at equal amounts per well and uptake of phages was measured after 2 h of incubation using a phage binding assay. (A–C) Binding of selected phages to different cell types: 5G6, 5E5 and 2G3, respectively (black bars). Hatched bars: library (unselected phage pool), white bars: wild-type (WT) phage (VCS-M13). 3T3: murine fibroblasts, H5V: murine endothelial cells, AT3 murine hepatoma cells, RAW: murine monocytes macrophages. Each bar represents the mean ± S.E.M. from three independent experiments. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ obtained by ANOVA.

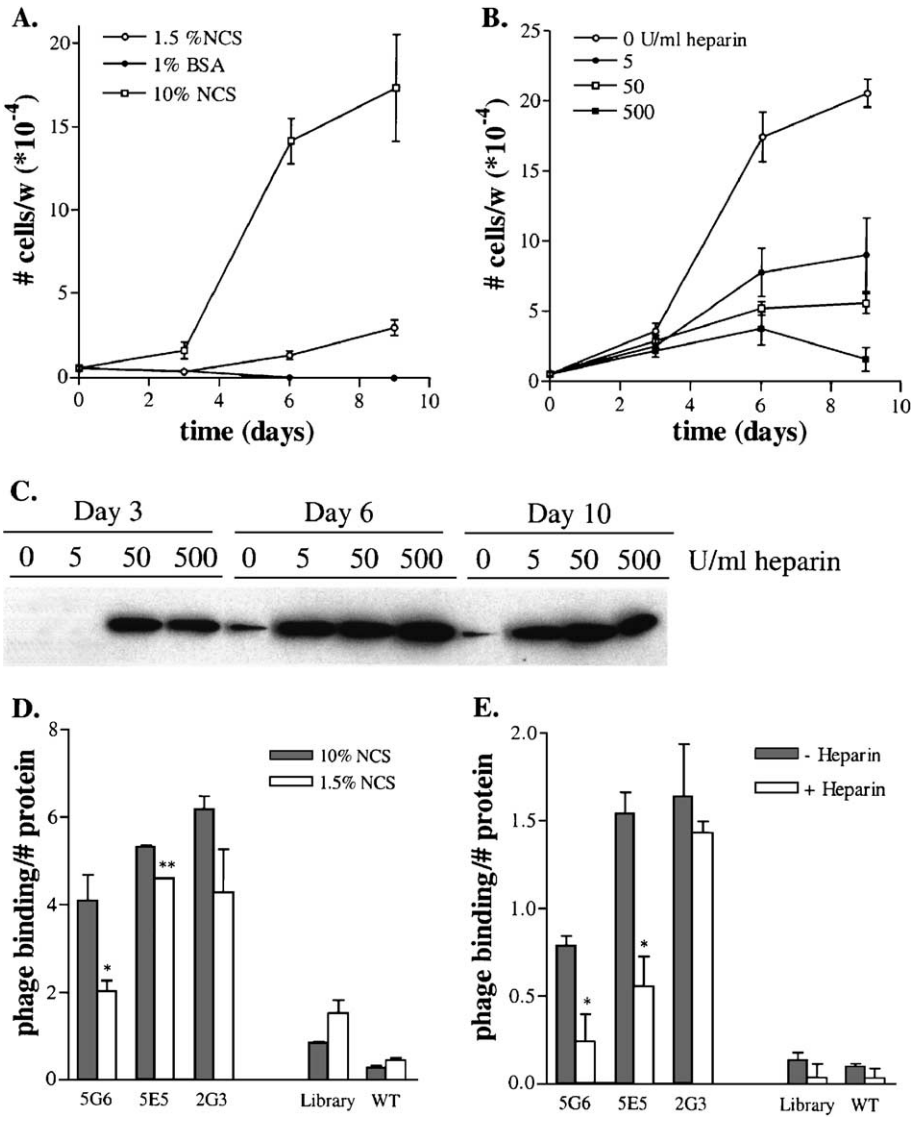

Fig. 5. Binding of selected phages to proliferating and non-proliferating VSMC. (A) Growth of VSMC at different serum levels, (B) growth of VSMC after the addition of heparin, (C) western blot of VSMC (8 μg protein/lane), stained for α-SM-actin, (D) binding of selected phages to proliferating VSMC (grey bars) and low-serum proliferation inhibited VSMC (white bars), (E) binding of selected phages to proliferating VSMC (grey bars) and heparin-proliferation inhibited VSMC (white bars). Binding was measured by a phage binding assay. Subsequently, phage binding was corrected for cell amounts by expressing binding per amount of protein produced per well. Bars represent average ± S.D. (duplicates). * $p<0.05$ and ** $p<0.01$, obtained by Student's *t*-test.

clones and phage binding was assayed on confluent monolayers. To correct for possible differences in cell numbers that will occur after several days of culturing, protein levels were also determined. Subsequently, phage binding was expressed as binding per amount of protein. The results are shown in Fig. 5. All clones except 2G3 bound with significant preference to proliferating VSMC compared to heparin-inhibited VSMC. Similar results were obtained with serum-starved VSMC, though the differences in binding between proliferating and heparin-inhibited cells are less apparent. Differences in binding of phages to heparin-inhibited or serum-starved VSMC are difficult to explain or to compare, because heparin is a multifunctional protein, which is also able to trigger many other cell functions and pathways.

3.5. Uptake of selected phages by VSMC

Binding of the selected phages to VSMC was visualized by growing VSMC on glass coverslips, incubation with phages and staining with biotinylated anti-M13 serum and FITC-labeled streptavidin. Most phages show a granular, lysosomal staining, whereas the consensus sequence 5G6 seems to be more concentrated around the nucleus. None of the selected phages binds to heparin-inhibited VSMC (Fig. 6).

To examine whether phages do not just bind to the surface of VSMC, but are also internalized by these cells, we examined the uptake of phages after the addition of some inhibitors of cellular uptake. Sucrose causes osmotic imbalance, resulting in disturbance of clathrin-coated pit forma-

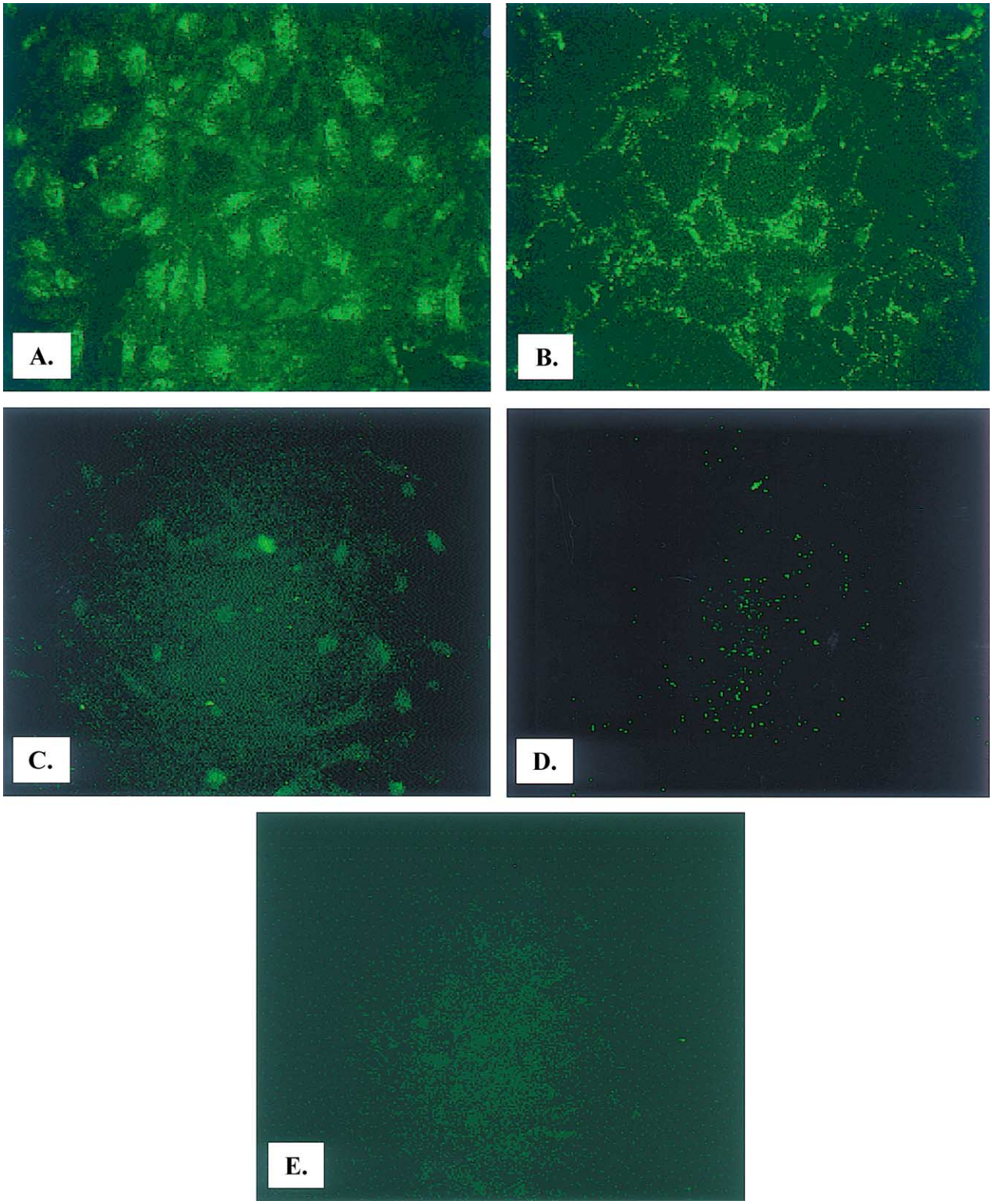

Fig. 6. Immunolocalization of selected phages on VSMC. VSMC were plated onto glass cover slips and incubated with phages. Phages were stained using biotinylated anti-M13 antibody and streptavidin-FITC (Amersham). Proliferating (A–C) and heparin growth inhibited VSMC (D,E) incubated with 5G6 phages (A,D), 2G3 phages (B) and wild-type phages (C,E) respectively. Magnification $\times 200$.

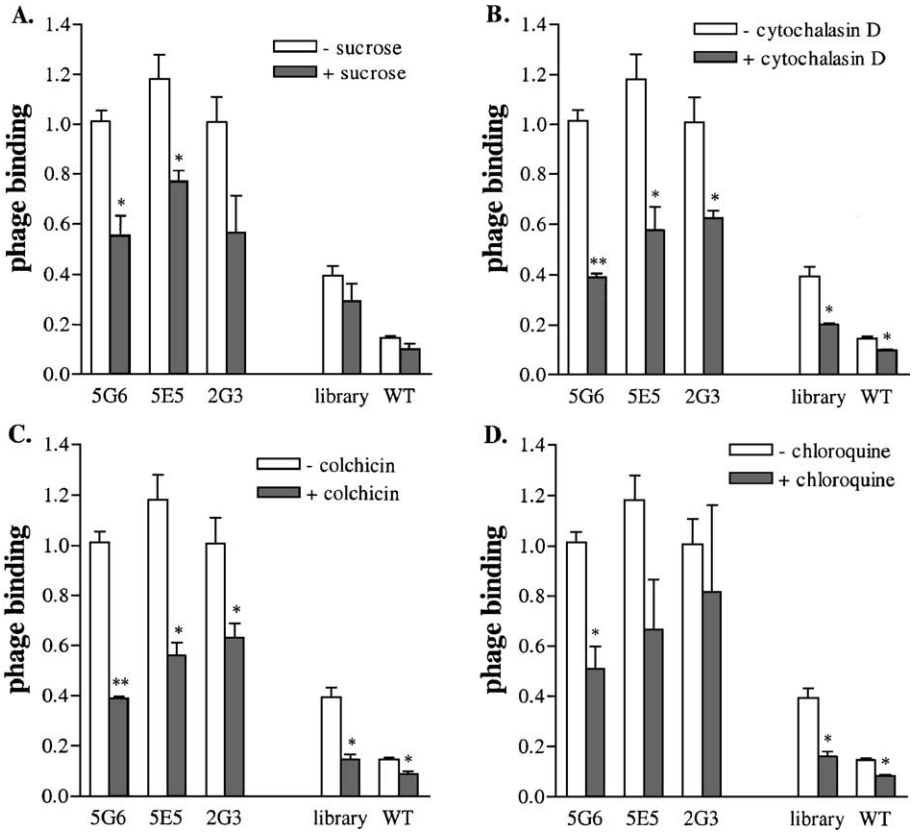

Fig. 7. Inhibition of phage uptake by VSMC by different inhibitors. Inhibition of phage uptake by 0.2 M sucrose (A), 10 μM cytochalasin D (B), 10 μM colchicin (C), 100 μM and 100 μM chloroquine (D). Phage binding was measured using phage binding assay. Bars represent average ± S.D. (duplicates). * $p<0.05$ and ** $p<0.01$, obtained by Student's *t*-test.

tion and subsequently receptor-mediated uptake. Colchicin is an inhibitor of microtubili-dependent transport, whereas cytochalasin D is an inhibitor of actin-dependent transport. Chloroquine inhibits the lysosomal pathway by lowering the pH within the lysosomes. The results are shown in Fig. 7. All three phages were inhibited in uptake by three or more of the tested inhibitors, indicating that the phages are not just binding to the surface of VSMC, but are readily taken up.

4. Discussion

Intimal lesion formation after angioplasty is characterized by the hyperplasia of VMSC. Previously it has been demonstrated that inhibition of proliferation and migration of this cell type by cytostatic drugs or adenoviral therapy can lead to diminished lesion formation [3,4]. However, there is still a need for an effective delivery system that can accomplish local delivery of drugs after systemic administration. For restenosis, a ligand that targets proliferating VSMC may greatly enhance the efficacy of drugs that inhibit proliferation of VSMC.

In this study, we isolated phages from a random, 15-mer constraint peptide phage display library that bind to proliferating VSMC. We used both in vitro and in vivo selection methods. In vitro biopanning did not result in a real consensus sequence. However, a recurring motif could be identified, like FLGW and LASR. After subsequent in vivo selection in mice, two consensus phages, 5E5 and 5G6 (Table 2), could be identified. These sequences were selected both in vitro and in vivo, but predominantly in vivo. Also, a clear recurring motif, IGR, could be detected in all sequenced clones (Table 1). Interestingly, consensus motifs between phages selected via biopanning on living cells or upon injection in vivo, often appear to be three or four amino acid sequences [23,25,30,31]. In many phage display studies involving selection of peptides on living cells, like in vivo selection for skeletal muscle-binding peptides [24] or in vitro selection on primary airway epithelial cells [32], no consensus motif between the selected phages can be detected at all. The identification of a consensus motif is limited by the variation within the library, as well as the length of the peptides encoded by the library (15 amino acids), as not all possible combinations will be present. The longer the encoded peptides, the less possibilities will be present due to limited variation possibilities of bacterial libraries. Therefore, libraries encoding short peptides, for example, 6-mer libraries are more likely to give rise to consensus motifs between selected peptides

than 12- to 20-mer libraries. Selection of phages on intact cells as compared to selection using a defined protein adds another important factor that reduces the chance of finding consensus sequences, since peptides selected for a certain cell type will bind to several targets, thereby scattering the number of possible motifs even more. This scattering seems to be dependent on the cell type or organ used for targeting. Clearly, the expression of proteins with high ligand binding capacities such as adhesion molecules or other cell-binding proteins plays an important role, as well as their expression levels on the cell type of interest. In vivo selection is even more complicated, as this selection method is also highly dependent on the vascularization of the target organ [24,33]. This way, it is more likely to end up with a consensus sequence rather than a consensus motif. To find all possible or improved motifs for cell binding, random mutation of the consensus sequence has proven to be [20].

Analysis of amino acid composition of the selected phages reveals that all selected sequences are relatively apolar, with few positively charged amino acids. Blast analysis showed no exact matches. However, significant homologies (70% or more positive) between most of the VSMC-selected phages and several protein groups could be distinguished. A large group of VSMC selected phages shares significant homologies with viral proteins and signaling proteins, indicating that the selected phages might be able to incorporate into the cellular membrane and enter the target cell likewise. Also, homologies with ion transport channels and G-protein-coupled receptors were listed, proteins known for their large trans-membrane domains. Another important group of phages shows high homology with structural proteins like gelsolin and integrins, proteins known to play an important role in VSMC and plaque formation in restenosis. For example, one of the phages that were selected both in vitro and in vivo, 5E5, matches a 10-amino-acid-region within the extracellular part of integrin α_5 of three different species (mouse, human and bovine). Together with the integrin β_1 subunit, this integrin is known as the fibronectin receptor, also called VLA-5 or CD49e. Interestingly, nine of the phages we selected, including the consensus 5G6 phage, share three amino acid homologies with a peptide specific for an organ that merely consists of SMC, the uterus-specific peptide, GLSGGRS [25].

The consensus phages 5G6 and 5E5 bound with a high selectivity to VSMC compared to endothelial cells and liver cells. Binding to fibroblasts, a cell type that shares high homology with proliferating VSMC, was also lowered compared to VSMC binding. However, a non-specific uptake by macrophages was not abolished. Furthermore, all selected phages tested bind preferentially to proliferating VSMC compared to VSMC allowed to differentiate by serum starvation or heparin. The results indicate that the phages we selected, not only are specific for VSMCs, but also for a specific physiological state of these cells. This specific phenotype of VSMC is generally believed to play an important role in the pathogenesis of restenosis [1]. Also, our studies with inhibitors of cellular uptake showed that all selected phages were effectively internalized by the target cells.

Though using cultured cells for biopanning is more easy and consistent, it is well known that the in vitro situation can be quite different from the in vivo situation. Therefore, we decided to select in vivo for restenotic VSMC targeting peptides as well, using an established mouse denudation model [28], in which the endothelial cells were removed using a rough guidewire, to expose the VSMC. In vivo phage display has proven to be very effective in selecting phages with high organ specificity upon systemic injection. However, these studies are based on the fact that molecular heterogeneity of the vascular endothelium can serve as addresses for each organ. Endothelial cells are specialized in homing of blood cells and humoral factors to the vascular wall. For this purpose, they express a high number adhesion molecules like selectins, ICAM, VCAM-1 as well as several types of differentially expressed proteoglycans and integrins [33,34]. In our case, selection occurred at the site of early-phase restenosis, with lesions predominantly composed of proliferating and migrating VSMC. To our knowledge, there are no unique surface markers expressed uniquely on VSMC, specialized in uptake of blood-derived factors, which may make them more difficult to target.

Previously, we have shown that chemical modification of wild-type M13 phages can alter plasma half-life and biodistribution of phages upon systemic injection. Conjugation of phages with galactose and succinic anhydride resulted in a significantly reduced plasma half-life, due to rapid and specific uptake by the liver and spleen [35]. For in vivo phage display, this implies that phages within a library expressing ligands that cause rapid internalization, might not reach their target at all. This will inevitably result in a phage population that binds to the endothelium predominantly. This is illustrated by the fact that all previous in vivo selections [23,25] and even an ex vivo study in denudated rat carotid artery [36] using a naive phage library, resulted in phages that preferentially bind to endothelial cells. Only the group of Samoylova and Smith [24], who used cultures of primary skeletal muscle cells to pre-select their phage library, was able to recover peptides that target skeletal muscle upon systemic injection. Also, previous attempts by our group to select phages that bind to cells within vascular lesions upon systemic injection in mice, using a naive phage library, failed (unpublished results). To avoid that most phages during in vivo selection would be cleared by adherence to endothelial cells before reaching the site of interest, we preselected them on primary cultures of VSMC, enhancing the number of copies per phage for VSMC targets. Furthermore, we chose to select for a longer time period than previous studies, which are merely based on 4- to 15-min systemic exposure, to avoid phage internalization. We allowed the phages to circulate for 2 h, to be able to

recover phages internalized by VSMC. To our knowledge, this is the first study in which selection of phage binding to VSMC over endothelial cell binding is established.

To investigate the in vivo targeting capacity of the selected phages, each clone was re-infused into the described restenotic mouse model. We observed accumulation of phages in organs that are predominantly responsible for the uptake of control phages (i.e. wild-type phages), such as liver and spleen (unpublished observation). This may be a result of the fact that only a limited number of the coat protein VIII expressed by the phage carry the recombinant peptide. We think that using the isolated peptide in our future in vivo research will resolve this problem.

In conclusion, these results show that the selected peptide sequences can be used for targeting to sites that are rich in proliferating VSMC, like restenotic lesions. When coupled to a drug, it may cause an enhanced and more efficient uptake of the drug into lesions and into the target cells when administrated systemically. The other way round, identification of the receptor to which the selected peptides bind to, might also result in the discovery of a VSMC-specific receptor. Until now, no cell surface receptor specific for VSMC is identified yet. Both the peptide and the receptor can be used for drug or gene targeting, as well as imaging studies.

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