Binding and retention of polycationic peptides and dendrimers in the vascular wall

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Abstract **Extracellular matrix (ECM) of tissues, vascular tissue in particular, contains a high concentration of negatively charged glycosaminoglycans (GAGs), which are involved in the regulation of cell motility, cell proliferation and the regulation of enzyme activities. Previously, we have shown that the vascular ECM is capable of binding an extremely high concentration of positively charged molecules, such as polylysine. Vascular ECM can be used therefore as a substrate for binding and retention of drugs delivered intravascularly, if these drugs are endowed with an ability to bind to the vascular ECM. In this study, we evaluated a number of positively charged molecules as potential affinity vehicles for delivery of drugs to the vascular ECM. We labelled the molecules of interest with fluorescence and compared them ex vivo in terms of binding and retention in the de-endothelialised rat carotid artery after intravascular delivery under pressure. High molecular weight polylysine (84 kDa) and polyamidoamine (PAMAM) dendrimers accumulated in the wall of the artery up to a concentration of 10 mg/ml and were not washed away significantly after 4 h of perfusion of the artery. A 24-mer peptide containing a consensus sequence for binding to GAGs $(ARRRAARA)_3$, 2.7 kDa, was comparable to high molecular weight polylysine and dendrimers in terms of binding and retention. A 14-mer GAG-binding peptide from vitronectin and low molecular weight polylysine, 3 kDa, accumulated in the vascular wall up to about 3 mg/ml and was washed away after 30 min of perfusion. A 10-mer consensus GAG-binding peptide did not bind significantly to the vascular tissue. We conclude that the consensus 24-mer GAG-binding peptide is by far superior to polylysine of a similar molecular weight in terms of binding to vascular tissue, and can provide high accumulation and long-term retention of a low molecular weight compound (fluorescein, as a model molecule) in the vascular wall. Rationally designed GAG-binding peptides can be useful as affinity vehicles for targeting drugs to the vascular ECM.**

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Key words: Drug delivery; Extracellular matrix; Glycosaminoglycan; Heparin-binding; Peptide design

1. Introduction

Extracellular matrix (ECM) of the vascular tissue represents an attractive target for therapeutic intervention. Proteoglycans are ubiquitous components of the ECM; their glycosaminoglycan (GAG) chains are involved in many biological functions, including regulation of cell motility, cell growth, and tissue morphogenesis [1]. GAGs represent about 2–5% of the non-aqueous material of the vascular wall [1], and consist of a negatively charged branched network with a profound capacity for binding various molecules including growth factors, factors involved in blood coagulation, lipid metabolism etc. Most of these GAG-binding proteins contain so-called GAG-binding consensus sequences of amino acids, identified by Cardin and Weintraub [2] mainly on the basis of molecular modelling. These sequences contain clustered patterns of positive charge XBBXBX or XBBBXXBX, where B represents a basic amino acid, and X represents an uncharged or hydrophobic amino acid. Fromm et al. [3] have found experimentally that a slightly different sequence is optimal for binding of short peptides to GAGs. In particular, among 10-mer peptides containing a structure RRG_mRR or $RRRG_mR$ the peptide GRRRGGGRGG shows the highest binding to heparan sulphate.

In a previous paper, we have shown that high molecular weight, positively charged molecules, like polylysine and polyamidoamine (PAMAM) dendrimers, can be accumulated in the vascular tissue up to the concentration of 10 mg/ml and retained within the vascular wall in vivo up to several days [4]. This offers a possibility of using positively charged GAG-binding molecules as affinity vehicles for drug targeting. Such an approach could work in combination with local intravascular delivery via catheter-based devices or via eluting stents [5,6].

The possibility of using highly charged polylysine-like molecules for in vivo applications, like gene targeting and others [7,8], is being actively investigated. However, in vivo applications of polylysine are likely to be limited because the extremely high charge is associated with toxicity [9,10]. For the purpose of drug targeting to GAG-containing ECM, polypeptides comprising GAG-binding consensus sequences may be a better choice, since they have a lower average charge density.

In this paper, we compare a number of molecules including polylysine, dendrimers, and a number of consensus GAG-

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binding peptides in terms of their binding and retention in a model of intravascular delivery. We show that a 24-mer, but not a 10-mer, consensus peptide is comparable to large polylysine and dendrimer molecules in terms of intravascular binding and retention.

2. Materials and methods

2.1. Materials

Fluorescein isothiocyanate (FITC), fluorescein-5-maleimide, *N*-succinimidyl-*S*-acetylthioacetate (SATA), were from Pierce. Poly-L-lysine (3 kDa and 84 kDa), as well as bovine albumin, were obtained from Sigma. Starburst™ PAMAM dendrimers (generations 4 and 5) were purchased from from Aldrich. The peptides were synthesised by Eurosequence, Groningen, The Netherlands. A peptide GRRRGGGRGG-amide containing one consensus sequence for binding to GAGs [2,3] and the GAG-binding 14-mer peptide from vitronectin with the sequence KKQRFRHRNRKGYR [11] were both synthesised with an additional cysteine on the N-terminus. The long consensus sequence peptide $(ARRRAARA)_3$ [12] was synthesised with an additional alanine at the N-terminus, modified with SATA, which introduces a protected SH-group. A random 12-mer peptide GPYSGEPKPFSA flanked with cysteine on the N-terminus was used as a negative control.

2.2. Labelling with fluorescein derivatives

Polylysines and dendrimers at a concentration of 5 mg/ml in a buffer containing 30 mmol/l sodium bicarbonate and 140 mmol/l NaCl, pH 9.2, were incubated for 1 h in the dark with FITC at a final concentration of 250 μM. The cysteine-containing peptides (the short consensus peptide and the GAG-binding peptide from vitronectin) at 0.5 mM in Tris-buffered saline (TBS), containing 1 mM EDTA, were incubated with fluorescein-5-maleimide at 1 mM for 1 h, and unreacted groups were blocked with 5 mM *N*-ethylmaleimide. The SATA-containing long consensus sequence peptide was labelled with fluorescein-5-maleimide under the same conditions after deprotection of SH-groups (incubation with 0.5 M hydroxylamine HCl for 2 h, according to a Pierce protocol). To separate the unbound dye from the labelled molecules we used gel filtration on Sephadex G-25 for large molecules (polylysine 84 kDa and dendrimers), whereas for the peptides we used either CM–Sepharose ion-exchange chromatography with elution by a gradient of NaCl, or reverse-phase high-performance liquid chromatography.

2.3. Ex vivo intravascular delivery of labelled molecules into rat carotid artery

A segment (approximately 1.2 cm) of the common carotid artery was removed from an anaesthetised rat and kept for not longer than 1 h in colourless RPMI cell culture medium at 4°C before the experiment. The labelled molecules were delivered into such a segment,

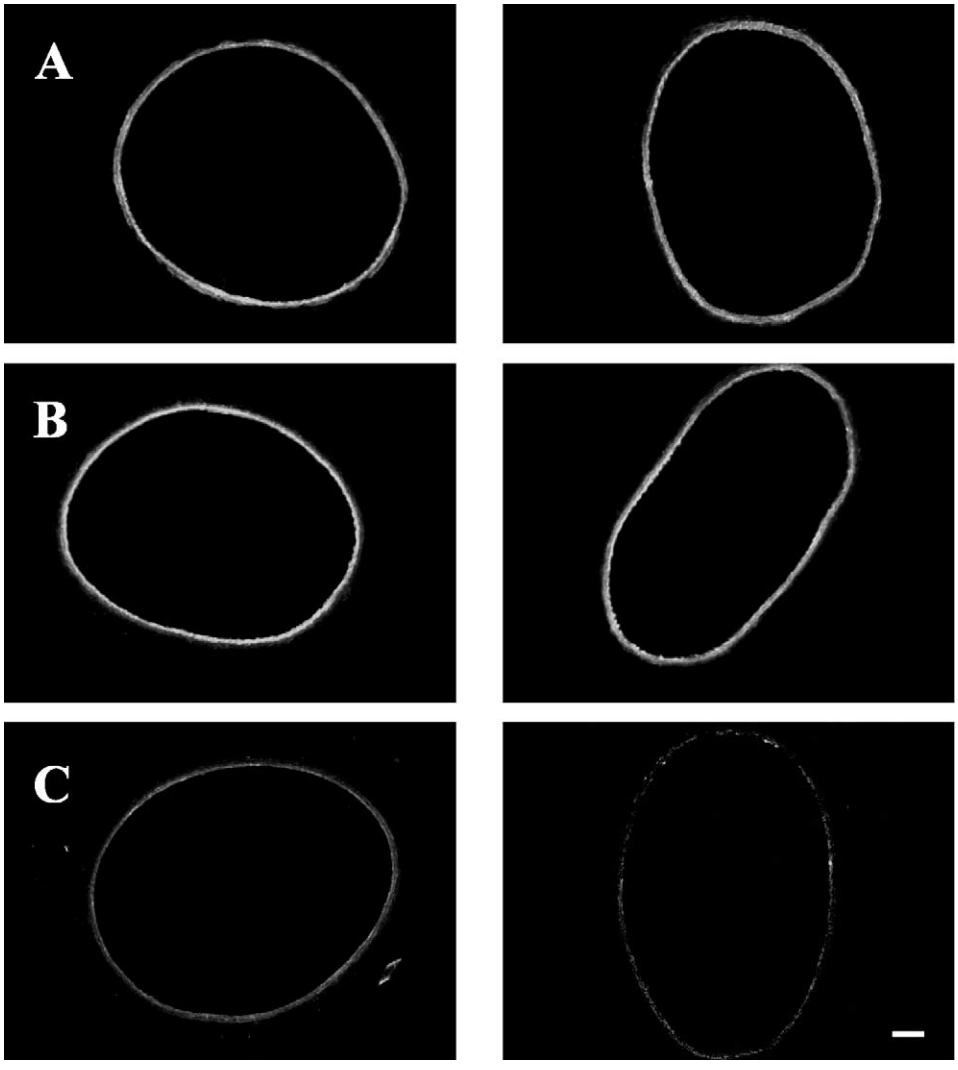

Fig. 1. Binding and retention of fluorescein-labelled molecules in the rat carotid artery after intravascular delivery under pressure. Fluorescence images of 20 μm thick cross-sections of arteries. A: Starburst dendrimer, generation 5, immediately after delivery (left) and after 4 h of perfusion with cell culture medium (right). B: consensus peptide $(ARRRAARA)_3$ immediately after delivery (left) and after 4 h of perfusion (right). C: 3 kDa polylysine immediately after delivery (left) and after 30 min of perfusion (right). Bar = 100 μm.

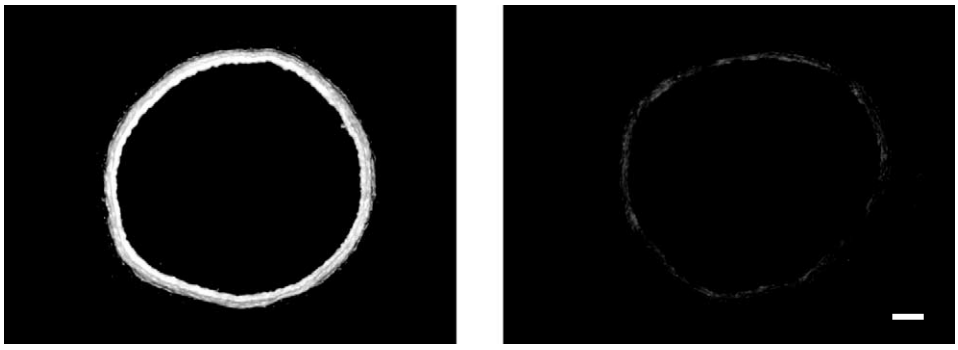

Fig. 2. Elution of bound fluorescein-labelled consensus peptide, $(ARRRAARA)_3$, from 20 μm thick cross-sections of a rat carotid artery. After intravascular delivery of the peptide under pressure, 20 μm thick cross-sections of the artery were prepared and eluted for 10 min either with PBS (left) or with phosphate buffer containing 2 M NaCl. Bar = 100 μm.

using the technique and conditions described elsewhere [4]. Briefly, one end of the vessel was secured on a plastic tube connected to a needle attached to a syringe equipped with a manometer. To remove any remaining blood and to destroy the endothelium, the vessel was rinsed with a few millilitres of RPMI and pumped through with air for 5 min with a peristaltic pump. Then, the vessel was filled in with RPMI, containing a labelled compound at 1 mg/ml, the open end of the vessel was closed with an atraumatic vascular clamp, and a pressure of 0.35 atm was applied for 20 min. Then, the clamp was removed, the vessel was briefly washed with RPMI, the distal half of the vessel was removed and snap-frozen. The proximal part of vessel, still connected to the needle, was perfused with RPMI through a peristaltic pump. After the desired time periods, the proximal half of the vessel was cut off and snap-frozen in a similar way.

2.4. Visualisation and quantification of the bound molecules

From the frozen pieces of the vessels, 20 μm cross-sections were prepared, dried and viewed under a fluorescence microscope. In order to quantify the local concentration of fluorescence-labelled molecules within the vascular wall, the sections were eluted with TBS, to which NaCl had been added to a final concentration of 2 M. The amount of fluorescence in the eluate was measured with an LS50B spectrofluorometer (Perkin-Elmer), and the amount of eluted fluorescence-labelled compound was determined using a calibration curve obtained with dilutions of the compound. The local concentration of bound fluorescence-labelled compound was calculated as the ratio of the amount of eluted compound and the volume of the section of the vessel, which was typically saturated with fluorescence throughout the whole depth of the vessel wall. The volume was calculated as a product of the thickness of the section (20 μm), the perimeter of the vessel and the width of the vessel wall. For each compound, the experiments were performed in duplicate. From each specimen corresponding to a particular perfusion time, at least six sections were prepared and used for quantification of binding.

3. Results

Using the model of intravascular delivery under pressure [4], we studied binding and retention of the following molecules in the vascular wall of the de-endothelialised rat carotid artery: high and low molecular weight polylysine (84 kDa and 3 kDa, respectively); PAMAM Starburst dendrimers of generations 4 and 5 (14 and 28 kDa, respectively); a 24-mer peptide, containing three copies of a consensus sequence for binding to GAGs, $(ARRRAARA)_3$; a 10-mer consensus peptide, GRRRGGGRGG; a 14-mer GAG-binding peptide from vitronectin, KKQRFRHRNRKGYR; and a control random 10-mer peptide.

Fig. 1 shows typical examples of the distribution of intravascularly delivered molecules in the wall of the rat carotid artery. PAMAM dendrimer, generation 5, and low molecular weight polylysine (3 kDa) exemplify molecules exhibiting, respectively, strong and weak binding to the vascular wall. The dendrimer bound to the tissue strongly and the amount of bound dendrimer remained virtually unchanged after 4 h of perfusion. The initial binding of low molecular weight polylysine was much weaker, and it was completely washed out already after 30 min of perfusion. The 24-mer consensus peptide, $(ARRRAARA)_3$, although a smaller molecule than polylysine, exhibited much stronger binding and retention, comparable to those of the dendrimer.

Most of the bound fluorescent material could be eluted from the sections by incubation of the sections with 2 M NaCl (Fig. 2). By measuring the amount of fluorescence eluted from the sections, we could quantify the amount of fluorescent molecules bound to the vascular wall and follow the kinetics of the wash-out of these molecules out of the vessel wall during 4 h of perfusion (Fig. 3). Consistent with our previously published data [4], 84 kDa polylysine and PAMAM dendrimer (generation 5, 28 kDa) were accumulated in the vascular wall up to a concentration of about 10 mg/ml and

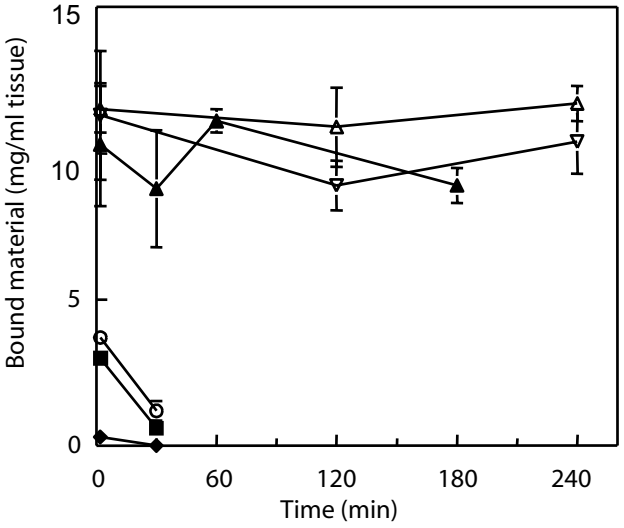

Fig. 3. Binding and retention of fluorescein-labelled molecules in the rat carotid artery. After intravascular delivery under pressure, the vessels were perfused with cell culture medium for the indicated periods of time. Bound fluorescence was eluted from the cross-sections of the artery and quantified in a spectrofluorometer. 84 kDa polylysine (△), PAMAM dendrimer generation 5 (▽), long consensus peptide $(ARRRAARA)_3$ (▲), GAG-binding peptide from vitronectin (■), 3 kDa polylysine (○), short consensus peptide GRRRGGGRG-amide (♦).

were not noticeably washed out after 4 h of perfusion. PAMAM dendrimer generation 4 (14 kDa) behaved essentially similarly (data not shown). The 10-mer consensus peptide did not accumulate at all in the vascular wall, similarly to a random 12-mer control peptide (data not shown). The 14-mer GAG-binding peptide from vitronectin and 3 kDa polylysine showed moderate accumulation (about 3 mg/ml) immediately after intravascular delivery, but after 30 min of perfusion they were completely washed out. Finally, the 24-mer consensus peptide $(ARRRAARA)_3$, 2.7 kDa, showed binding and retention comparable to those of high molecular weight polylysine and dendrimers.

4. Discussion

Local intravascular delivery of drugs is an active area of research in interventional cardiology and vascular medicine. Either local drug delivery catheters or eluting stents can be used for topical delivery of a drug to a desired segment of a blood vessel [5,6]. Efficiency of local delivery, penetration and distribution of the drug in the vascular wall, and retention after delivery are important parameters being studied both experimentally and theoretically [4,13–15]. Binding of a drug to components of the vascular wall is important for both increasing the efficiency of delivery (i.e. the fraction of the drug which leaves the catheter or eluting stent and is deposited in the vessel wall) and prolongation of the drug retention in the vascular wall. In our previous experimental and theoretical studies, we have shown that molecules incapable of binding to the vascular wall will be washed out by the bloodstream in a matter of minutes [4,15]. Some hydrophobic molecules, the potential anti-proliferative agent paclitaxel in particular, have been shown to interact with insoluble elements of vascular tissue, which results in prolonging the retention after delivery via an eluting stent [16]. Hydrophilic molecules, peptides and proteins in particular, are also being examined as potential drugs [13,17,18]. We have shown that positively charged hydrophilic molecules (polylysine, in particular) will be retained in the vascular wall for many hours after local delivery both in an ex vivo perfusion system and in vivo after restoration of the bloodstream [4]. Cationic derivatives of antioxidant enzymes, obtained by coupling the enzymes to polylysine, were also shown to be retained firmly in articular tissue and to suppress inflammatory response in an animal model of arthritis [19]. Tissue proteoglycans, which contain negatively charged GAG chains, are the most likely binding counterparts for cationic macromolecules.

In vivo applications of polylysine are limited due to its toxicity [9,10]. Modification of polylysine by partially blocking its positive charge with various groups has been shown to reduce its toxicity [7,20]. Synthetic polycationic dendrimers are being intensively studied for the same type of applications [21]. Dendrimers are highly branched spherical macromolecules with all bonds emanating from the central core. The size of dendrimer is determined by its generation number, i.e. the number of repetitions of a two-step reaction used for its synthesis. Although the dendrimers have a number of attractive features for applications in pharmaceutical chemistry, they still exhibit a considerable toxicity [22]. Using positively charged consensus peptides, which imitate naturally occurring GAG-binding sequences, is an alternative approach.

In the present paper, we studied how relatively short consensus GAG-binding peptides bind to and are retained within vascular tissue. Papers published earlier by others on the interaction between consensus peptides and GAGs (mostly heparin) either describe interaction of peptides with GAGs in terms of elution of the peptides from heparin–Sepharose with increasing ionic strength, or assess the K_d of interaction in buffers with non-physiologically low ionic strength [3,23]. In a recent paper of Verrecchio et al. [12], the interaction of consensus peptides with heparin was studied by affinity co-electrophoresis in a buffered sodium acetate solution. Here, we looked at binding of charged peptides directly to the vascular tissue using a model of intravascular delivery under pressure in rat carotid artery.

The results indicate that short peptides, containing a single GAG-binding consensus sequence, do not bind noticeably to the vascular tissue. In contrast, a peptide containing a linear trimer of a consensus sequence demonstrates an amount of binding and retention comparable to those of high molecular weight polycations (polylysine and dendrimers). Polylysine of the same molecular weight (3 kDa) exhibited a much weaker binding, indicating that consensus peptides indeed have an optimal spatial charge distribution providing maximal binding to GAGs in tissues. Our results are consistent with affinity co-electrophoresis data of Verrecchio et al. [12], who have shown that for consensus peptides XBBBXXBX each step from monomer to dimer and from dimer to trimer results in a drastic increase in affinity to heparin. Further increase in length of the consensus peptides (to 4- and 5-mers) resulted in only moderate if any further increase in affinity [12].

In conclusion, this paper demonstrates that relatively low molecular weight oligomers of GAG-binding consensus peptides bind considerably to the vascular tissue, and can therefore potentially be used as affinity vehicles for drug delivery.

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