Polylysine as a Vehicle for Extracellular Matrix–Targeted Local Drug Delivery, Providing High Accumulation and Long-Term Retention Within the Vascular Wall

D.V. Sakharov, A.F.H. Jie, M.E.A. Bekkers, J.J. Emeis, D.C. Rijken

Abstract—We present the first steps in the elaboration of an approach of extracellular matrix–targeted local drug delivery (ECM-LDD), designed to provide a high concentration, ubiquitous distribution, and long-term retention of a drug within the vessel wall after local intravascular delivery. The approach is based on the concept of a bifunctional drug comprising a “therapeutic effector” and an “affinity vehicle,” which should bind to an abundant component of the vessel wall. The aim of the present study was to select molecules suitable for the role of affinity vehicles for ECM-LDD and to study their intravascular delivery and retention ex vivo and in an animal model. By use of fluorescence microscopy, the following molecules were selected on the basis of strong binding to cross sections of human vessels: protamine, polylysine, polyarginine, a glycosaminoglycan-binding peptide from vitronectin, and a synthetic dendrimer. With polylysine as a prototypic affinity vehicle, we showed that after intravascular delivery, polylysine was concentrated throughout a luminal layer of the vascular wall to an extremely high concentration of 20 g/L and was retained therein for at least 72 hours of perfusion without noticeable losses. Low molecular weight (fluorescein) and high molecular weight (hirudin) compounds could be chemically conjugated to polylysine and were retained in the vessel wall after intravascular delivery of the conjugates. In conclusion, by use of the ECM-LDD method, an extremely high concentration and long-term retention of locally delivered drug can be reached. Polycationic molecules can be considered as potential affinity vehicles for ECM-LDD. (Arterioscler Thromb Vasc Biol. 2001;21:943-948.)

Key Words: local drug delivery ▪ restenosis ▪ polylysine ▪ hirudin ▪ extracellular matrix

Local drug delivery (LDD) is a useful approach for treating many localized pathologies. The concentration of systemically administered drugs is often limited by side effects of the drug. With LDD, a much higher local concentration of a drug in the site of pathology can be achieved. Treatment/prevention of restenosis after coronary angioplasty is currently one of the most tantalizing targets for intravascular LDD. A number of sophisticated catheter-based devices have been developed to maximize the efficiency of intravascular LDD; some of these devices do not obstruct the blood flow and, therefore, allow prolonged local delivery.1 The major problem of the intravascular drug delivery is that after being delivered to the site of pathology, the drug may be washed out from the site of delivery rather rapidly, over minutes to hours.1,2 Even if the drug interacts with the cells within the vascular wall, only a small fraction of the locally delivered drug would be caught up by the cells; the rest would be rapidly washed away. The fraction of the drug taken up by the cells may be either internalized or shed from the cellular membrane within hours or, at most, days. It would be desirable to keep a drug at the site of potential restenosis for a longer period of time, because restenosis is a long process, the initial phase of which takes a few weeks, and final changes develop after a few months.1,3

Incorporation of the drug into microparticles or hydrogels with slow (controlled) drug release is suggested as an approach for prolonging drug retention. However, the vessel wall appears to be an anatomic barrier to microparticle delivery; the particles penetrate poorly into the vascular wall and are found mostly in the vasa vasorum and dissections within the intima after angioplasty.4 A variant of controlled drug release is endoluminal paving with a drug-containing hydrogel.5 In this technique, the storage of the drug (hydrogel coating) is also located next to the target tissue but is not in it; thus, the drug, after being released from the hydrogel compartment, must penetrate the neighboring vascular tissue. Simulations show that this technique prolongs the intravascular presence of the drugs from minutes to hours, but not longer.6

Besides LDD, another alternative to systemic drug treatment is cell-targeted drug delivery. In this approach, focused mostly on the treatment of cancer,7 a systemically administered drug, endowed with an affinity to a pathology-specific epitope exposed on the cells, is supposed to find the site of
pathology and concentrate there as a result of binding to these epitopes. The approach is most attractive for the cases in which the location of pathology is not precisely known and the local delivery of the drug is not possible. Normally, a highly specific antibody should be generated to ensure the specific delivery of the drug to the targeted cell population in the presence of a great excess of irrelevant tissue epitopes. No reliable restenosis-specific epitopes have been identified as yet, so nowadays, this approach is not applicable to the prevention/treatment of restenosis.

In the present study, we report a novel approach of extracellular matrix (ECM)-directed local drug delivery (ECM-LDD), designed to provide a long-term retention of a highly concentrated drug within the target tissue after local delivery. The method is based on a concept of local delivery of a bifunctional drug comprising an anti-restenotic effector moiety and an “affinity vehicle” moiety capable of anchoring and retaining the drug within the ECM of the vessel wall. A principal feature of the approach, which distinguishes it from other drug-targeting and LDD systems, is that not pathology-specific epitopes but abundant structural components of tissue are used as a molecular target. Because such components are present throughout the tissue at high concentrations, the tissue would be used as a high capacity support for accumulating a high concentration of the drug.

In the present study, we describe a selection of molecules potentially suitable for the role of affinity vehicle within the aforementioned ECM-LDD approach.

Methods

Materials
Fluorescein isothiocyanate (FITC), fluorescein-5-maleimide, tetramethylrhodamine-5-(6)-isothiocyanate (TRITC), N-succinimidyl S-acetylthioacetate (SATA), and N-(maleimidocaproyloxy)-succinimide ester (EMCS) were from Pierce. Poly-L-lysine and poly-L-arginine of different molecular weights, ranging from 3000 to 99000, as well as lactoferrin and bovine albumin were obtained from Sigma Chemical Co. Protamine was from Kabi, and Starburst (PAMAM) dendrimer (generation 4) was from Aldrich. A peptide from vitronectin and the peptide containing the consensus sequence from vitronectin and the GAG-binding 14-mer (PAMAM) dendrimer (generation 4) was from Aldrich. A peptide kindly provided by Dr K. Preissner, Institute of Biochemistry, University of California, Irvine, and lipoprotein lipase from bovine milk was obtained from Boehringer, Mannheim, Germany.

In Vivo Intravascular Delivery of FITC-Polylysine Into Human Umbilical Artery

One end of a 2-cm piece of human umbilical artery was secured on a plastic tube connected to a syringe equipped with a manometer. To remove any remaining blood, the vessel was rinsed with a few milliliters of TBS and pumped through with a few milliliters of air. Then it was rinse with 200 μL of human citrated plasma containing 0.5 mg/mL FITC-polylysine. The open end of the vessel was ligated, and the same plasma with FITC-polylysine was pumped in with a syringe and kept under pressure of 0.35 atm for 30 minutes. Then the ligation was removed, and the vessel was perfused with TBS at 10 mL/min. After the desired time periods, pieces of the distal part of the vessel (=0.7 cm long) were cut off and snap-frozen in liquid nitrogen. A similar experiment was performed with FITC-labeled BSA. Cross sections (8 μm) of the vessel were dried and viewed under a fluorescence microscope.

In order to quantify the local concentration of FITC-polylysine bound within the luminal layer, 20-μm cross sections of the umbilical artery were prepared, and the bound FITC-polylysine was eluted from it with TBS, to which NaCl had been added to a final concentration of 2 mol/L. The amount of fluorescence in the eluate was measured with an LS50B spectrofluorometer (Perkin-Elmer), and the amount of eluted FITC-polylysine was determined by using a calibration curve obtained with dilutions of FITC-polylysine. The local concentration of bound FITC-polylysine was calculated as the ratio of the amount of eluted FITC-polylysine and the volume of the saturated layer of the section. The volume was calculated as a product of the thickness of the section, the perimeter of the vessel, and the depth of the layer saturated with polylysine. The experiment was performed in triplicate. At least 4 sections from each of the 3 vessels were used for FITC-polylysine quantification.

In Vivo Intravascular Delivery of FITC-Polylysine Into a Segment of Rat Carotid Artery

Male Wistar rats were anesthetized with pentobarbital sodium (Nembutal) and Hypnorm (0.315 mg fentanyl citrate and 10 mg fluanisone per milliliter), and a 1-cm segment of a common carotid artery was separated with atraumatic vascular clamps, freed of blood through a catheter introduced via the inner carotid artery, and washed with saline buffered with 20 mmol/L HEPES, pH 7.5. Then the segment was filled with either FITC-polylysine (99 kDa) or FITC-albumin, both at a concentration of 1 mg/mL, in the same buffer, and incubated for 20 minutes under a pressure of 0.35 atm (the pressure was limited by the strength of the clamps and was several-fold lower than the pressure achievable in intravascular catheters). Then the tip of the catheter was removed, and the bloodstream was restored. The rats were euthanized after timed intervals ranging from 15 minutes to 72 hours. The treated segments of the carotid arteries were removed and snap-frozen. The fluorescence retained was assessed with a fluorescence microscope and 8-μm frozen cross sections. Some of the frozen pieces of the vessels were thawed and fixed in ice-cold 10% formalin, paraffinized, and sectioned for histological staining with hematoxylin-phloxine-saffron.

Conjugation of Polylysine With Hirudin

The heterobifunctional cross-linking agent EMCS was used for conjugation of polylysine with recombinant hirudin. Hirudin (M, 7000) at 1 mg/mL was first incubated with 1.5 mmol/L SATA for 1 hour to introduce protected sulfhydryl (SH) groups into the hirudin molecule, and unreacted SATA was removed by gel filtration. Polylysine (99 kDa) at 1 mg/mL was incubated for 1 hour with 1.5 mmol/L EMCS to introduce SH-reactive maleimide groups into N-ethylmaleimide, and separated from unbound fluorescein by gel filtration into TBS.

Selection of Candidate Affinity Vehicles: Binding to Cross Sections of Blood Vessels

The fluorescein-labeled candidate affinity vehicles at a concentration of 30 μg/mL in human citrated plasma were incubated with 8 μm fresh-frozen sections of human aorta for 1 hour at room temperature, and binding was assessed by using a fluorescence microscope.
polylysine. After removal of free EMCS from the polylysine solution (gel filtration) and deprotection of SH groups in SATA-hirudin (incubation with 0.5 mol/L hydroxylamine HCl for 2 hours, according to a Pierce protocol), the 2 solutions were mixed at a molar ratio of 10:1 (hirudin:polylysine) and incubated for 1.5 hours, and cysteine was added to a final concentration of 30 mmol/L to block reactive groups. The hirudin-polylysine conjugate was separated from free hirudin on a column with CM-Sepharose, which was equilibrated with a 50 mmol/L phosphate buffer containing 50 mmol/L NaCl, pH 7.5. Free hirudin passed through, whereas the conjugate was eluted with a phosphate buffer containing 1.3 mol/L NaCl and dialyzed into saline buffered with 20 mmol/L HEPES, pH 7.5. The hirudin activity test showed that ~30% of hirudin was conjugated with polylysine. Thus, ~3 hirudin molecules were conjugated to each polylysine molecule.

Ex Vivo Intravascular Delivery of Hirudin-Polylysine Into Rat Carotid Artery
A segment (~1.5 cm) of the common carotid artery was removed from an anesthetized rat and kept for 12 to 18 hours in cell culture medium at 4°C before the experiment. The polylysine-hirudin conjugate at a concentration 1 mg/mL was delivered into such a segment by using the technique and conditions described above for human umbilical arteries. The segments of the arteries were then perfused with TBS for 20 minutes and frozen, and 8-μm cross sections were prepared. The sections were washed for 20 minutes with several changes of TBS containing 20 mg/mL BSA (TBS-BSA) and incubated with TRITC-thrombin at 10 μg/mL in TBS-BSA for 30 minutes. After the unbound fluorescence was washed away, the distribution of bound TRITC-thrombin was documented with the fluorescence microscope.

Results
Selection of Potential Affinity Vehicles: Binding to Cross Sections of Human Aorta
To select molecules suitable for the role of affinity vehicle for ECM-targeted LDD, we studied the binding of a number of candidate molecules to fresh-frozen sections of human aorta. Incubation with the sections was performed in the presence of human plasma to imitate the conditions of delivery in vivo. The following molecules were included in the study on the basis of their known GAG-binding properties: protamine sulfate, a peptide with a consensus sequence for binding to glycosaminoglycans, lactoferrin, lipoprotein lipase, a GAG-binding peptide from Aβ-amyloid, and a GAG-binding peptide from vitronectin. Polylysine and polyarginine of various molecular weights and Starburst dendrimer were included in the screening as well, because their strong positive charge suggests that they could also bind to vascular GAGs. Of the molecules tested, only a few showed a strong binding: namely, protamine sulfate, Figure 1A; polylysine (29, 52, and 99 kDa), shown in Figure 1B for polylysine 99 kDa; polyarginine (11 kDa), not shown; a GAG-binding peptide from vitronectin, Figure 1C; and the Starburst PAMAM dendrimer, Figure 1D. These molecules bound to all parts of the vessel wall, although the patterns of their staining showed certain difference. Polylysine of 10 kDa showed a somewhat weaker binding (not shown), whereas high molecular weight polyarginine (92 kDa) caused protein aggregation in plasma. The other molecules tested did not bind to the sections: namely, the peptide with a consensus sequence for binding to glycosaminoglycans, lactoferrin, lipoprotein lipase, a GAG-binding peptide from Aβ-amyloid, polylysine with a molecular weight of 3000 (not shown), and albumin (Figure 1E).

Delivery and Retention of Affinity Vehicle Within Human Umbilical Artery Wall Ex Vivo
Human umbilical artery was used as a simple ex vivo model of a middle-sized human artery to study intravascular delivery and retention of polylysine. After 30 minutes of delivery...
under pressure, FITC-polylysine was accumulated in a sharply visible luminal layer of ≈80 μm depth (Figure 2A). The bound material was not washed out noticeably after 72 hours of perfusion (Figure 2B). In contrast, FITC-BSA did not accumulate significantly in the vessel wall and was completely washed out after 1 hour (Figure 2C).

To estimate the local concentration of FITC-polylysine bound within the luminal layer, the bound material was eluted from 20-μm-thick sections with a buffer containing 2 mol/L NaCl. The greater part of the fluorescence was removed from the cross section as a result of such elution. Amounts of FITC-polylysine in the range of 140 to 260 ng were eluted from the sections with a standard variation between different sections within the same vessel not >6% (n=4). The volumes of the fluorescence-bearing areas ranged from 7 to 10 nL. On the basis of 3 independent experiments, the concentration of the bound polylysine within the stained area was calculated as 21.9±3.8 g/L (n=3).

Delivery and Retention of Affinity Vehicle in Wall of Rat Carotid Artery In Vivo
After intravascular delivery in vivo into a rat carotid artery and restoration of the blood flow through the vessel, bound FITC-polylysine was found throughout the vessel wall (Figure 3A). After 24 hours and 72 hours (Figure 3B and 3C, respectively), a significant part of originally bound fluorescence could still be found throughout the vessel wall. In contrast, FITC-albumin was mostly washed away in 90 minutes (Figure 3D). Histological examination of the sections of rat carotid arteries removed 24 hours (Figure 3E and 3F) and 72 hours (not shown) after delivery of FITC-polylysine or FITC-albumin revealed neither significant changes in the morphology of the vessel wall nor signs of inflammation, immune response, or thrombosis. The state of the endothelium was not ideal in either FITC-polylysine–treated or FITC-albumin–treated vessels, probably as a consequence of the intravascular intervention or of the freezing and thawing of the vessels before they were embedded in paraffin.

Delivery of Hirudin Into Rat Carotid Artery Ex Vivo
Figure 4 shows that hirudin coupled to polylysine can be found within a luminal layer of the artery after intravascular delivery ex vivo. Staining with TRITC-labeled thrombin was used to visualize the distribution of the bound hirudin within the vessel wall and to demonstrate that bound hirudin was still functional. The penetration of the conjugate into the vascular wall was somewhat restricted compared with that of polylysine (Figure 3A), supposedly because of the larger molecular size of the conjugate. If a mixture of the 2 unconjugated components (hirudin and polylysine) was delivered into the vessel wall in a similar manner, almost no binding of TRITC-thrombin to the cross sections was observed (not shown).

Discussion
In the present study, we describe a novel approach in drug delivery, ECM-LDD. The major idea behind this approach,
distinguishing it from other drug-targeting and LDD methods, is that structural extracellular components of tissue are used as a high-capacity support capable of accumulating a high concentration of the drug. The bifunctional drug, comprising an affinity vehicle and a “therapeutic effector” moiety, is delivered under pressure into a target tissue and is retained by binding of the affinity vehicle to an abundant and ubiquitous component(s) of the ECM, providing long-term retention of the drug in the lumen of pathology. The most interesting application of the approach is, obviously, prevention/treatment of restenosis. Therefore, the present study is focused on the application of ECM-LDD to intravascular delivery.

An important feature of the ECM-LDD is that the affinity vehicle does not have to show pathology-specific binding, because the drug would be directed to the desired site not by site/pathology-specific molecular recognition but by local delivery under pressure via an intravascular catheter. Strong binding of the affinity vehicle to the target component(s) and also the abundant and ubiquitous presence of the target component(s) within the vascular wall are seen as essential requirements for the successful realization of the ECM-LDD approach.

The present study is focused on a selection of potential affinity vehicles that meet these requirements and on a characterization of their local intravascular delivery and retention. Molecular targets for ECM-directed delivery are the major proteins of the vascular ECM (eg, collagen, laminin, elastin, and fibronectin) and GAGs. Vascular GAGs are distributed throughout all layers of the vessel wall rather ubiquitously and, therefore, represent an attractive target for drug delivery. We screened a number of molecules with known GAG-binding properties, as well as a few positively charged molecules, which might bind to the negatively charged GAGs.

Selection

Of a number of compounds screened, a few showed significant binding to cross sections of human aorta in the presence of plasma. Polylysine with an $M_\text{s}$ of $\approx$29,000 showed the most even and ubiquitous binding in this assay and, therefore, was used in further experiments. A number of other molecules, such as protamine, the GAG-binding peptide from vitronectin, and a Starburst dendrimer, were also positive in the selection assay and are currently being studied in more detail. The rest of the molecules did not bind to the sections, although on the basis of the data from the literature, binding might have been expected. Among them are the short peptide with a consensus sequence for GAG binding. The lack of binding of such peptides is consistent with the recent data by Verrecchio et al., who report that 3- and 4-mers of such peptides, but not monomers and dimers, show strong binding to GAGs.

Characterization

With polylysine as a model affinity vehicle, we made the following observations, important for characterizing this method of drug delivery: (1) After intravascular delivery under pressure, bound polylysine is found in a luminal layer at a depth of a few 10s of micrometers. In small vessels (as rat carotid artery), this results in saturation of the whole depth of the vessel wall; in larger vessels (as human umbilical artery), only a part of the vessel is saturated with bound polylysine. (2) Within the said layer, bound polylysine is distributed evenly and ubiquitously, showing no preferential staining of any particular structures. Such distribution is consistent with the hypothesis that polylysine binds mostly to GAGs, which are present throughout the vascular wall. (3) On binding within the luminal layer, polylysine concentrates up to 20 g/L. This gives an estimate of the concentration of the drug that can be delivered and retained in the vascular wall using the method of ECM-LDD. (4) Polylysine bound within the luminal layer of the vessel wall is retained for at least days ex vivo under perfusion conditions and in vivo after restoration of the blood flow.

All these observations are consistent with the idea that the binding sites within the luminal layer of the vascular wall become saturated with polylysine as a result of strong binding and that the layer, saturated with polylysine, slowly extends along with pressure/diffusion-driven influx of polylysine from the lumen. Polylysine concentrates within this layer because of a high concentration of the binding sites.

An important implication of the aforementioned observations is that in addition to increased retention, the ECM-LDD method may increase the efficiency of local delivery, ie, the fraction of the substance that leaves the catheter and is deposited in the vessel wall. In practice, this value is generally $<1\%$. Because of strong binding to the ECM, polylysine molecules entering the vascular wall appear to be captured therein and, therefore, are prevented from escaping to the systemic circulation. It has been shown recently that the efficiency of intramural delivery of platelet-derived growth factor and tissue factor pathway inhibitor, which both have moderate binding properties toward the extracellular matrix, is somewhat higher than that of nonbinding proteins. However, the saturation-like profile has not been observed in the case of platelet-derived growth factor, probably because the binding was not strong enough. Tissue factor pathway inhibitor was mostly found next to the luminal surface. How strong binding to ECM (eg, in the case of polylysine) affects the efficiency of catheter-based intravascular delivery is a subject of our further study. We are also studying in a greater detail how the concentration of bound affinity vehicle, the depth of its penetration into the vascular wall, and retention are affected by properties of the affinity vehicle (size, charge, and affinity) and those of the target component (concentration and accessibility of the binding sites) as well as by the variables of local delivery (pressure and duration) and the state of endothelium.

Accessibility of the subendothelial ECM is an important condition for realization of the ECM-LDD. In the case of anti-restenosis treatment, the method is supposed to be used in situations in which the endothelium is either destroyed or damaged by ballooning and preexisting atherosclerosis. In the present study, we did not physically remove the endothelium (although it could be damaged, because in all experiments a certain amount of air was bubbled through the vessels, and the vessels used in ex vivo experiments were stored for certain time at 4°C before the experiments). Still, this did not prevent accumulation of polylysine within the vessel wall, suggesting that the method can potentially be used also in situations in which the endothelium is not fully removed. This is consistent with the data showing that endothelium repre-
Comparison of Existing Methods of Drug Administration With the Method of ECM-LDD

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<th>Potential Advantages</th>
<th>SDA</th>
<th>CTDD</th>
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<td>No need for pathology-specific epitope</td>
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SDA indicates systemic drug administration; CTDD, cell-targeted drug delivery; and CDR-LDD, controlled drug release after LDD.

sent only a relative barrier for the transport of macromolecules, reducing transmural transport of middle-sized proteins 4- to 7-fold, and that the transendothelial transport of proteins increases severalfold as a result of pressure application.

In the present study, we have demonstrated that the example affinity vehicle (polylysine) can deliver either a small molecule (fluorescein) or a relatively large biologically active polypeptide (hirudin) to the ECM of the vascular wall. Thus, in general, low molecular weight compounds and proteins/enzymes can be tested in the future as therapeutic effectors within the ECM-LDD approach. The list of potential therapeutic effectors includes anticoagulants, antiplatelet drugs, cytostatics, cytoskeletal inhibitors, proapoptotic effectors, vasorelaxants, and other substances considered as potential anti-restenotic drugs for systemic or local administration. Depending on the desirable mode of action (eg, extracellular or intracellular), the therapeutic effector may be attached to the affinity vehicle either through a stable or an unstable link, respectively, providing, in the latter case, a controlled release of the drug inside the vascular wall.

The Table summarizes important features of the existing variants of drug administration compared with the ECM-LDD method. It is to be hoped that this method will open new perspectives in intravascular drug delivery, because it provides a combination of extremely high local concentration, uniform and ubiquitous distribution, and long-term intravascular retention of the drug, apparently unachievable with other methods.

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References

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