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Molecular vehicle for target-mediated delivery of therapeutics and diagnostics

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Abstract

Selective targeting of therapeutic and diagnostic agents improves their efficacy and minimizes potentially adverse side effects. Existing methods for selective targeting are based on chemical conjugation of therapeutics and diagnostics, or their carriers, to cell-specific targeting molecules (e.g., growth factors, antibodies). These methods are limited by potential damage to targeting molecules that can be inflicted by the conjugation procedure. In addition, conjugation procedures have to be developed on a case-by-case basis. In order to avoid these problems we have developed a new approach to constructing molecular vehicles for target-mediated delivery of therapeutics and diagnostics. In this approach, the targeting molecule is expressed as a fusion protein containing a *recognition tag*. The recognition tag is defined as a peptide or protein that can bind non-covalently another peptide or protein (*adapter*). In turn, the adapter is chemically conjugated to a *carrier* of therapeutics or diagnostics. The assembled molecular delivery vehicle contains a carrier–adapter conjugate bound non-covalently to a recognition tag fused to the targeting protein. The advantages of this technology are: (i) no chemical modification of targeting molecules, and (ii) universal, 'off-the-shelf' carrier–adapter constructs that can be combined with different fusion targeting proteins. To obtain a proof-of-principle we have constructed VEGF fusion proteins containing a 15-aa S-peptide fragment of RNase A as a recognition tag. Using the S-protein fragment of RNase A as an adapter and polyethylenimine as a DNA carrier we have achieved selective gene delivery to cells overexpressing VEGFR-2.

Keywords: Drug delivery; VEGF; Gene therapy; Angiogenesis; Endothelial cells

1. Introduction

Selective delivery of therapeutic, diagnostic, and research compounds to targeted cells improves their efficacy and minimizes potentially adverse side effects. Monoclonal antibodies, metabolites, peptide hormones, cytokines, growth factors, viral, and bacteriophage particles have been employed as targeting entities. Two main approaches have been used to load therapeutic, diagnostic, and research compounds onto these targeting entities (reviewed in Ref. [1]). In the first approach, molecules intended for delivery are chemically conjugated to the targeting molecules. Accumulated experience indicates that no more than five to 10 molecules can be conjugated to a single targeting molecule. In a second approach, a high capacity carrier for molecules intended for delivery is either chemically conjugated to the targeting molecule, or is bound non-covalently to a specific group introduced into the targeting molecule (e.g.,

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biotin). Several carriers have been introduced in practice, such as dextrans, synthetic polymers, and various liposomes.

Both approaches, described above, rely on chemical modifications of the targeting molecules and therefore have to be custom-developed and optimized on a case-by-case basis. Moreover, these approaches are limited by potential damage to targeting molecules that can occur during the conjugation procedure. In order to avoid these problems we have developed a new approach to construct molecular vehicles for target-mediated delivery of therapeutics and diagnostics. In this approach, the targeting molecule is expressed as a fusion protein containing a *recognition tag*. The recognition tag is defined as a peptide or protein that can bind non-covalently to another peptide or protein (*adapter*). In turn, the adapter is chemically conjugated to a *carrier* of therapeutics or diagnostics. The advantages of this approach are: (i) no chemical modification of targeting molecules, and (ii) universal, 'off-the-shelf' carrier–adapter constructs that can be combined with different fusion targeting proteins. To obtain a proof-of-principle for our approach, we have constructed $VEGF_{121}$ fusion protein containing a 15-aa S-peptide fragment of RNase A as a recognition tag. Using a 108-aa S-protein fragment of RNase A as an adapter and polyethylenimine as a DNA carrier we have achieved selective gene delivery to cells overexpressing VEGFR-2.

2. Materials and methods

2.1. Chemicals

Polyethylenimine (PEI, ~25 kDa) and S-protein fragment of RNase A (SP) were purchased from Sigma. S-peptide (S-tag) fragment of RNase A was purchased from Novagen. *m*-Maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (sulfo-MBS), 2-iminothiolane (Traut's reagent), and Ellman's reagent were purchased from Pierce. A reporter plasmid pRL-tk for expression of Renilla luciferase was obtained from Promega.

2.2. VEGF fusion proteins

$VEGF_{121}$ fusion protein containing an S-tag in the N-terminal fusion domain has been constructed, cloned, expressed in BL21(DE3)pLys5 *E. coli*, and purified as described elsewhere (Backer and Backer, submitted). The concentration of $VEGF_{121}$ was determined with an S-tag Rapid Assay Kit (Novagen) according to the manufacturer's protocol.

2.3. Cell lines

Transformed human primary embryonic kidney cells (293 cells, ATCC CRL-1573, American Type Culture Collection, Rockville, MD), and their derivatives expressing 2.5×10^6 full-length VEGFR-2 receptors/cell (293/KDR cells, developed at SibTech) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and antibiotics at 37°C, 5% CO_2. The ability of 293/KDR and 293 cells to accept and express foreign DNA was determined using DNA/PEI assembled with N/P ratio of 8 (N is a molar concentration of PEI amino groups, and P is a molar concentration of DNA phosphate groups). In every experiment, the transfectability of 293/KDR and 293 cells was found to be was similar within 15% experimental error (data not shown).

2.4. Preparation of PEI-SP conjugates

PEI (5 mM) was incubated with Trout's reagent (5 mM) for 20 min at room temperature under nitrogen in a buffer containing 50 mM triethanolamine, 150 mM NaCl, 1 mM EDTA. The product was purified on a dextran desalting column equilibrated with a buffer containing 0.1 M NaPi, pH 7.2, and 150 mM NaCl (conjugation buffer). The concentration of modified PEI was determined by the Ellman's reaction on free SH-groups according to the manufacturer's instructions. The content of PEI in each fraction was calculated as a fraction of the total Ellman's reactive material.

S-protein (0.66 mM) was incubated with sulfo-MBS (1.32 mM) for 30 min at room temperature in conjugation buffer. The product was purified on a dextran desalting column equilibrated with conjugation buffer. The content of modified S-protein in each fraction was calculated as a fraction of the total 280 nm absorbing material.

Modified S-protein (0.127 mM) was incubated with equimolar amount of modified PEI for 4 h at

room temperature in conjugation buffer. Conjugate (PEI-SP) was purified from unreacted S-protein on a G-100 Sepharose column equilibrated with phosphate-buffered saline. Fractions containing the peak of PEI-SP were combined, aliquoted, and stored at −20°C.

2.5. Preparation of DNA/PEI-SP-VEGF complexes

PEI-SP was mixed with $VEGF_{121}$ at the desired ratio in serum-free DMEM and the mixture was incubated on ice for 20 min. This mixture was then added to a chilled solution of pRL-tk in serum-free DMEM at the desired ratio and incubated for additional 20 min on ice.

2.6. Binding of DNA/PEI-SP-VEGF to cellular VEGFR-2 receptors

The ability of DNA/PEI-SP-VEGF to compete with ^{125}I-$VEGF_{165}$ for binding to cellular VEGFR-2 was assayed as described [2].

2.7. DNA delivery to cells

The protocol followed the procedure optimized for detection of VEGF-induced VEGFR-2 tyrosine phosphorylation [2]. 293 and 293/KDR cells were plated on 24-well plates at 10^5 cells/well. After 16–20 h media was changed to serum-free DMEM supplemented with 25 mM Hepes, pH 7.2, and cells were incubated for 4 h at 37°C, followed by a 20-min incubation at 4°C and addition of the ice-cold solution of DNA/PEI-SP-VEGF or DNA/PEI. After a 40-min incubation at 4°C cells were shifted to 37°C, and after a 4-h incubation DMEM was supplemented with 10% FBS. Luciferase activity was measured 24 h later. In each experiment, cells were transfected with a mixture of DNA and PEI at a N/P ratio of 8 as a control for transfectability. In separate experiments, DNA complexes were added directly to cells grown in DMEM supplemented with 10% FBS. All DNA delivery experiments were repeated 2–3 times. According to our data and in agreement with reports from other groups [3–6] luciferase expression varies several-fold between experiments reflecting, most likely, some subtle changes in the properties of DNA complexes [7].

2.8. Expression of luciferase in 293/KDR cells

Activity of luciferase in cell lysates was quantitated using a commercially available 'Dual-Luciferase Reporter Assay System' from Promega according to the manufacturer's instructions. In separate experiments with triplicate wells, variations in the levels of luciferase expression within the same experiment were less than 20%. We therefore used single wells for the majority of experimental points.

3. Results and discussion

A VEGF-based molecular vehicle for DNA delivery was constructed as shown in Fig. 1. The S-peptide and S-protein fragments of RNase A served as the recognition tag fused to VEGF, and adapter, respectively. Independently, these fragments do not display ribonuclease activity. However, they form a high affinity ($K_d \sim 10^{-9}$ M) catalytically active complex, known as RNase S, and studies of this system proceed for more than 40 years.

3.1. S-protein conjugated to PEI binds to S-tag in VEGF

S-protein was conjugated to PEI, a well-known DNA carrier, through a procedure that included (1) introduction of a single SH-group in PEI, (2) introduction of one to two maleimide groups in S-protein, and (3) crosslinking of modified PEI and S-protein via reaction between SH and maleimide groups. The ability of S-protein to bind S-peptide with restoration of ribonuclease activity was progressively decreased by modification with sulfo-MBS (Table 1). In experiments described below, we therefore employed S-proteins treated with a 2-fold molar excess of sulfo-MBS.

We found that PEI-SP formed catalytically active complexes with S-tag in $VEGF_{121}$ (Fig. 2). Interestingly, PEI-SP-VEGF displayed higher ribonuclease activity than SP-VEGF suggesting that PEI might

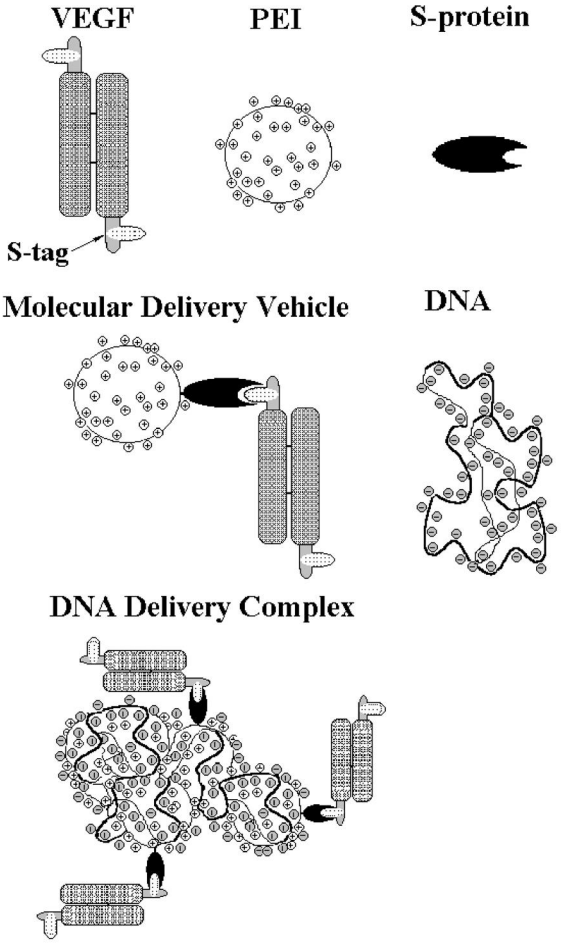

Fig. 1. Construction of VEGF-based molecular vehicle for gene delivery.

Table 1
Effect of modification of S-protein with sulfo-MBS on the reconstructed ribonuclease activity

SulfoMBS, molar excess	Ribonuclease activity[a] (%)
None	100
2	57
10	38
20	36
40	25

[a] Ribonuclease activity was measured with the S-tag Rapid Assay Kit (Novagen).

facilitate interactions between the substrate and the reconstructed RNase (Fig. 2).

3.2. DNA/PEI-SP-VEGF binds to cellular VEGFR-2

We found that DNA/PEI-SP-VEGF competed with ^{125}I-VEGF$_{165}$ for binding to VEGFR-2 in 293/KDR cells as efficiently as VEGF$_{121}$ (Fig. 3). We hypothesized that the simultaneous presence of several VEGF$_{121}$ molecules per DNA/PEI-SP-VEGF might enhance binding to cellular VEGFR-2 and compensate for any possible negative effects of bulky entities at the N-terminus of VEGF.

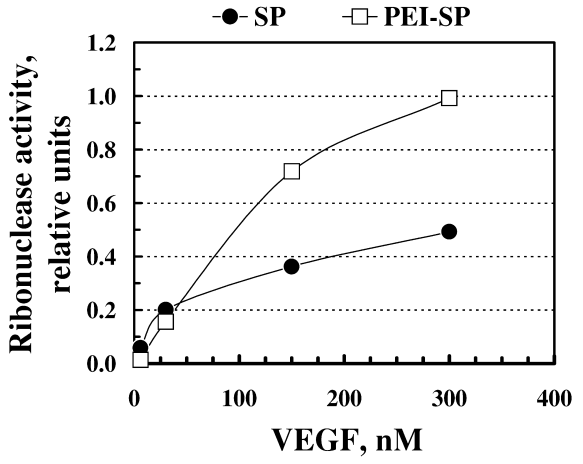

Fig. 2. PEI-SP conjugate binds to S-tag in VEGF fusion protein. Reaction mixtures contained 5 nM PEI-SP or S-protein (SP) and indicated concentrations of S-tagged $VEGF_{121}$. Ribonuclease activity was measured with S-tag Rapid Assay Kit (Novagen) according to the manufacturer's instructions. Ribonuclease activity is presented as the optical density of acid-soluble material formed after hydrolysis of polyC.

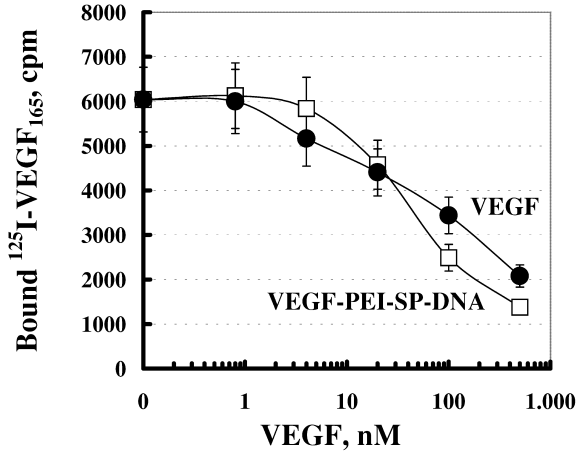

Fig. 3. DNA/PEI-SP-VEGF binds to cellular VEGFR-2 as avidly as $VEGF_{121}$. 293/KDR cells were plated on 24-well plates at 200 000 cells/well. Twenty-four hours later media were changed to serum-free DMEM supplemented with 25 mM Hepes pH 7.5. [^{125}I]$VEGF_{165}$ (40 000 cpm/well) was mixed with $VEGF_{121}$ or with DNA/PEI-SP-VEGF and added to triplicate PBS-washed cells at the indicated concentrations. After 90 min at room temperature media was removed and cells were washed twice with ice-cold PBS/1% BSA and once with ice-cold PBS/1%BSA/0.4 M NaCl. Cells were lysed with 2% Triton X-100, 0.25 M NaCl, 10 mM Tris–HCl and counted in a Beta-counter.

3.3. DNA/PEI-SP-VEGF selectively delivers DNA into 293/KDR cells

VEGFR-2-mediated DNA delivery by DNA/PEI-SP-VEGF was tested with 293/KDR cells expressing $\sim 2.5 \times 10^6$ receptor/cell. As a control, we have used parental 293 cells lacking VEGFR-2. Additional experiments demonstrated that 293 and 293/KDR cells are similar in their ability to accept and express foreign DNA. We have also used DNA/PEI-SP prepared with the same N/P ratio as DNA/PEI-SP-VEGF.

DNA/PEI-SP-VEGF and DNA/PEI-SP were incubated with 293/KDR and 293 cells in serum-free medium under conditions described in Section 2. The efficiency of DNA delivery was measured 24 h later by the levels of luciferase activity in targeted cells. DNA/PEI-SP complexes constructed with an N/P ratio of 3.75 (chosen to prevent electrostatic interactions between cell surface and DNA/PEI-SP) failed to deliver DNA into 293/KDR and 293 cells (Fig. 4A). In contrast, DNA/PEI-SP-VEGF with the same N/P ratio of 3.75 delivered DNA into both types of cells. However, DNA delivery into 293/KDR cells was greatly enhanced as compared to parental 293 cells (Fig. 4A). Depending on experimental conditions, DNA delivery into 293/KDR cells was 3–15 times higher than into parental 293 cells suggesting that a significant part of DNA entered these cells via a VEGFR-2-mediated process.

The DNA delivery experiments described above were performed under conditions optimized for detection of the cellular VEGFR-2 phosphorylation that are far removed from in vivo conditions (see Section 2). We therefore tested whether VEGFR-2-mediated DNA delivery may take place at more physiological conditions. We found that DNA/PEI-SP-VEGF delivered DNA into 293/KDR and 293 cells in a VEGF-dependent manner when cells were maintained at 37°C in DMEM supplemented with 10% FBS (Fig. 4B). Under these conditions, DNA delivery into 293/KDR cells was 5–20 times more efficient than in 293 cells (Fig. 4B).

The VEGF-mediated delivery of DNA into 293 cells, which do not express any known high affinity receptors for VEGF, suggested additional pathways of DNA entry. Although we employed $VEGF_{121}$ lacking a heparin-binding domain, it was possible

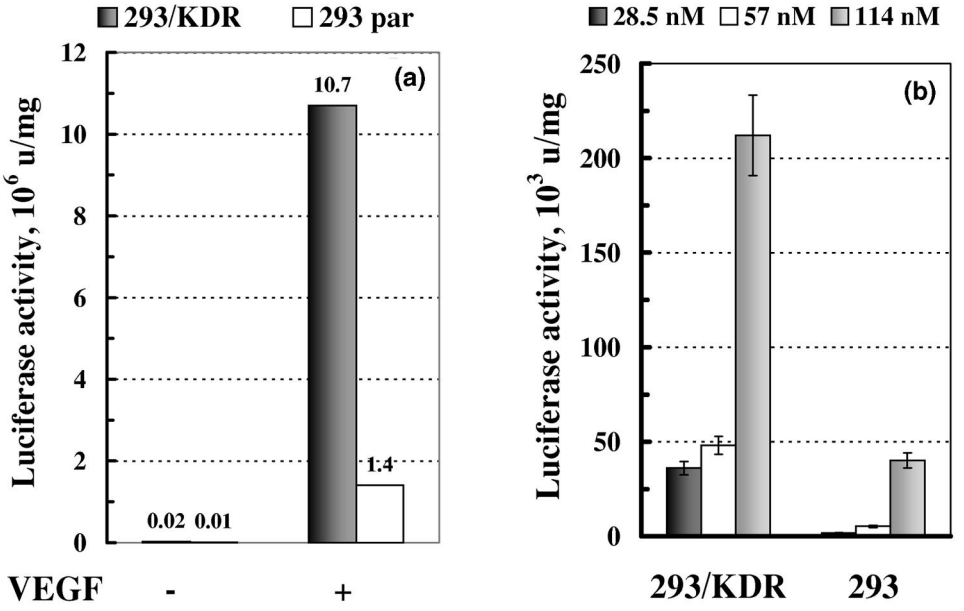

Fig. 4. VEGF-based molecular vehicle delivers DNA into cells overexpressing VEGFR-2. (A) DNA delivery in serum-free DMEM. DNA/PEI-SP-VEGF and DNA/PEI-SP prepared with the *N*/*P* ratio of 3.75 were added to cells preincubated at 4°C in a serum-free DMEM to final concentrations of 30 nM VEGF, 64 nM PEI-SP conjugate, and 2 μg/well DNA. After additional 40 min at 4°C, plates were shifted to 37°C and after a 4-h incubation supplemented with 10% FBS. (B) DNA delivery in DMEM supplemented with 10% FBS. DNA/PEI-SP-VEGF prepared with the *N*/*P* ratio of 3.75 were added to cells incubated at 37°C in DMEM supplemented with 10% FBS to final concentrations of 64 nM PEI-SP, 2 μg/well DNA, and indicated concentrations of VEGF. Luciferase activity was measured in cell lysates 24 h after DNA addition.

that DNA/PEI-SP-VEGF may still bind to cell surface proteoglycans via the PEI moiety. To test this hypothesis, DNA/PEI-SP-VEGF were bound to 293 and 293/KDR cells at 4°C and then washed with 0.4 M NaCl prior to incubation at 37°C. The salt wash decreased DNA delivery into 293/KDR cells only by ~20%, while delivery into 293 cells was diminished by 70% (Fig. 5). These results suggested that the majority of DNA enters 293/KDR cells via the VEGFR-2-mediated route. However, some DNA/PEI-SP-VEGF may enter 293/KDR and 293 cells via electrostatic interactions with cell surface proteoglycans, and deliver DNA into cells through this route.

3.4. Potential of technology

Our results provide evidence that VEGF-based molecular gene delivery vehicle can be constructed without chemical modification of VEGF. We believe that molecular vehicles for delivery of therapeutic and diagnostic agents may be constructed using other targeting proteins, different carriers, and new tag–adapter combinations. We expect that construction of a targeting protein that can be combined with the pre-made adapter–carrier conjugates would require less efforts than development of a customized chemical modification procedure for the same protein. Experiments are now in progress to apply our technology for delivery of various therapeutics and diagnostics.

Acknowledgements

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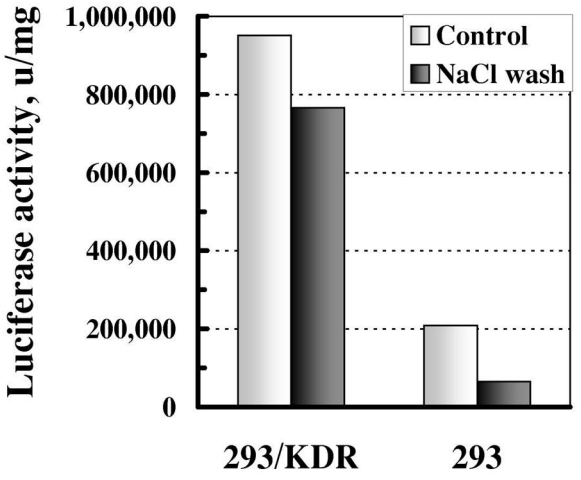

Fig. 5. VEGF-mediated DNA delivery into 293/KDR cells proceeds mostly via VEGFR-2-mediated pathway. DNA/PEI-SP-VEGF prepared with the *N*/*P* ratio of 7.5 were added to cells preincubated at 4°C in a serum-free DMEM to final concentrations of 57 nM VEGF, 32 nM PEI-SP and 0.5 μg/well DNA. After a 40-min incubation at 4°C, cells were washed once with 0.4 M NaCl, shifted to 37°C, and 4 h later supplemented with 10% FBS. Luciferase activity was measured in cell lysates 24 h after DNA addition.

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