Molecular Vehicles for Targeted Drug Delivery

Marina V. Backer,* Renee Aloise, Kristen Przekop, Konstantin Stoletov,† and Joseph M. Backer

SibTech, Inc., Newington, Connecticut 06111, and Albert Einstein College of Medicine, Bronx, New York 10461.
Received November 7, 2001; Revised Manuscript Received March 4, 2002

Targeted drug delivery by cell-specific cytokines and antibodies promises greater drug efficacy and reduced side effects. We describe a novel strategy for assembly of drug delivery vehicles that does not require chemical modification of targeting proteins. The strategy relies on a noncovalent binding of standardized "payload" modules to targeting proteins expressed with a "docking" tag. The payload modules are constructed by linking drug carriers to an adapter protein capable of binding to a docking tag. Using fragments of bovine ribonuclease A as an adapter protein and a docking tag, we have constructed vascular endothelial growth factor (VEGF) based vehicles for gene delivery and for liposome delivery. Assembled vehicles displayed remarkable selectivity in drug delivery to cells overexpressing VEGF receptors. We expect that our strategy can be employed for targeted delivery of many therapeutic or imaging agents by different recombinant targeting proteins.

INTRODUCTION

The current revolution in drug discovery is driven by functional genomics, proteomics, high throughput screening, and in silico drug design. It is widely expected that these new technologies will yield numerous potent and highly selective drugs. Targeted drug delivery, as opposed to systemic delivery, can dramatically increase drug efficacy while decreasing side effects and the patient's treatment burden. Targeted delivery requires "loading" drugs onto cell-specific cytokines or antibodies. Although initially these targeting proteins were purified from natural sources, now most of them are available as recombinant proteins. Thus, effective technology for loading drugs onto recombinant targeting molecules is crucial for channeling the current revolution in drug discovery into targeted drug delivery.

Currently, several strategies are used for loading drugs onto targeting proteins (*1*). In one approach, drugs (e.g., radionuclides) are directly conjugated to targeting proteins. In another approach, drugs are loaded onto high capacity drug carriers (e.g., liposomes, synthetic polymers, etc.). These carriers are either directly conjugated to targeting proteins or derivatized for interactions with specific adapters that are conjugated to the targeting proteins. Streptavidin/biotin interaction is particularly widely explored for binding various carriers to targeting proteins (*2*). Since both strategies rely on chemical conjugation of drugs, carriers, or adapters directly to targeting proteins, they share common problems such as (i) potential for inactivation of cell binding domains by conjugation, (ii) the inevitable heterogeneity of final products, and (iii) development of custom conjugation procedures for every targeting protein. These problems are the major stumbling blocks in using cell-specific proteins for targeted drug delivery. Recently, heterobifunctional recombinant antibodies recognizing one epitope on the cell surface and another epitope on the drug carrier have been proposed for targeted drug delivery (*3*, *4*). This approach avoids chemical modification of targeting antibody but it requires custom development of heterobifunctional recombinant antibodies, and the prospects of efficient internalization of antibody are uncertain.

We have recently proposed a new strategy for converting targeting proteins into vehicles for drug delivery (*5*). The strategy relies on a modular assembly of drug delivery vehicles and therefore avoids random chemical modification of targeting proteins. We proposed to construct standardized "payload" modules by linking various drug carriers to a special adapter protein (Figure 1). The function of the adapter protein is to bind a payload module to a "docking" tag fused to a targeting protein. In this approach, the assembly of a drug delivery vehicle does not require chemical modification of targeting proteins and relies on standardized components. Here we describe vascular endothelial growth factor (VEGF) based vehicles for delivery of liposome-encapsulated doxorubicin and for gene delivery. To construct these vehicles we have used a 104-aa fragment of bovine ribonuclease A (known as S-protein), as an adapter protein and a 15-aa fragment of bovine ribonuclease A (known as S-peptide) as a docking tag. This tag is incorporated in various pET expression vectors from Novagen (Madison, WI) and is widely used for affinity purification and quantitation of recombinant proteins. Assembled vehicles displayed remarkable selectivity in drug delivery to cells overexpressing VEGF receptors. We expect that our strategy can be employed for targeted delivery of many therapeutic or imaging agents by different recombinant targeting protein.

MATERIALS AND METHODS

Cells. 293 human transformed embryonic kidney cells (CRL-1573) and HL-60 human promyelocytic cells (CCL-240) were from American Type Culture Collection (Rockville, MD). PAE/KDR porcine aortic endothelial cells expressing 2.5×10^5 VEGFR-2/cell and PAE/V control cells were kindly provided by Dr. B. Terman (Albert Einstein School of Medicine, Bronx, NY). 293/KDR cells expressing 2.5×10^6 VEGFR-2/cell and PAE/0.1-KDR

* To whom correspondence should be addressed at SibTech, Inc., 705 North Mountain Road, Newington, CT 06111. Phone: (860) 953-1753; Fax: (860) 953-1317; E-mail: mbacker@sibtech.com.

† Albert Einstein College of Medicine.

10.1021/bc0155770 CCC: $22.00
Published on Web 04/12/2002

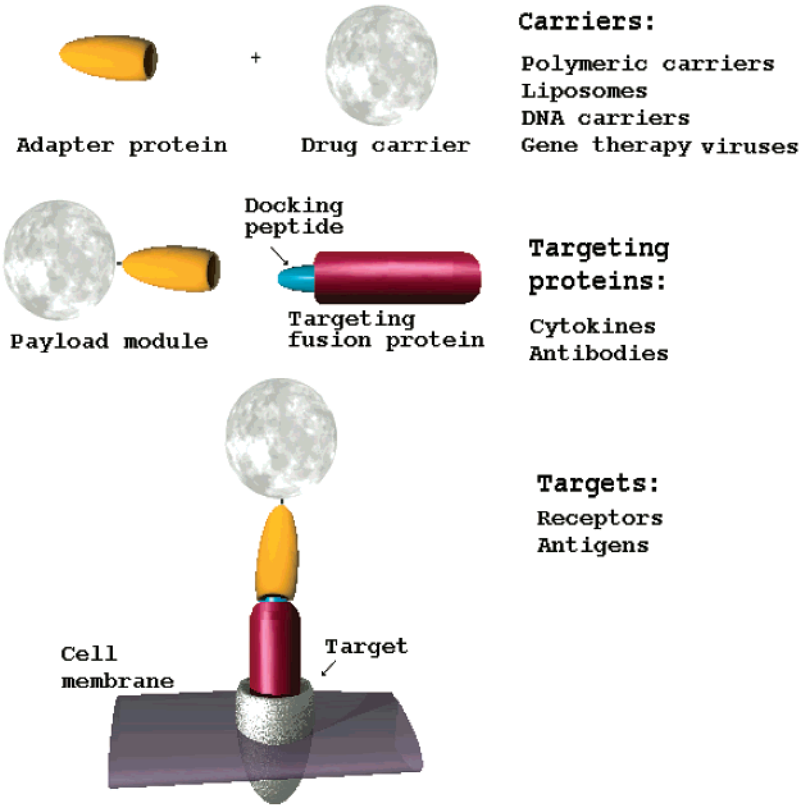

Figure 1. Construction of a molecular delivery vehicle.

expressing 2.5×10^4 VEGFR-2/cell have been developed in SibTech, Inc. (*6*, *7*). HL-60 cells were grown in RPMI 1640 (Gibco Life Technologies, Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS, Gemini, Newton, MA), 4 mM L-glutamine, and antibiotics at 37 °C, 5% CO_2. All other cells were grown in DMEM with 10% FBS, 2 mM L-glutamine, and antibiotics at 37 °C, 5% CO_2.

Plasmids. $VEGF_{121}$ was cloned into pET-32a(+) (Novagen, Madison, WI) containing a coding sequence for a 15-aa fragment of bovine ribonuclease A, known as S-tag, as described previously (*7*). DNA encoding *E. coli* thioredoxin was removed from pET-32/$VEGF_{121}$ plasmid by *Nde*I digestion. The resulting pET/$VEGF_{121}$ plasmid encoded human $VEGF_{121}$ with a 42-aa N-terminus containing S-tag. S-tagged VEGF was expressed in Origami-(DE3)pLysS (Novagen) and purified as described (*8*). The concentration of the purified protein was determined by the S-tag Rapid Assay kit (Novagen) and reflected the concentration of S-tagged VEGF monomers. Human procaspase 8b (Mch5-beta, GenBank accession: AF009620) was amplified by RT-PCR using total RNA of HL-60 cells (RNeasy RNA isolation kit, Qiagen, Valencia, CA) with primers 5′-ac*tctaga*ttggtcacttgaaccttggg and 5′-ac*tcta-ga*agcaccatcaatcagaaggg introducing *Xba*I sites (italic); and cloned into pcDNA3.1/Zeo(+) (Invitrogen, Carlsbad, CA). Cloned cDNAs were confirmed by sequencing. The pGL3 plasmid encoding firefly luciferase was from Promega (Madison, WI).

Liposomes Preparation and Loading. S-protein fragment of bovine ribonuclease A (SP, Sigma, St. Louis, MO) at a concentration of 0.93 mM was modified with 0.7 mM poly(ethylene glycol)-α-distearoyl phosphatidylethanolamine, ω-NHS ester (DSPE-PEG-NHS, average MW 3400, Shearwater Polymers, Huntsville, AL) in 0.1 M NaPi, 0.15 M NaCl, pH 7.2, under argon at 37 °C for 4 h. Dioleoyl phosphatidylcholine (Avanti, Alabaster, AL) and cholesterol (Sigma) were mixed at the molar percent ratio of 65:35, lyophilized from cyclohexane, and hydrated in a buffer containing 10 mM HEPES pH 7.2, 150 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, and 0.34 mM doxorubicin (Dox, Sigma, St. Louis, MD), and 13 μM DSPE-SP. After four freeze−thaw cycles, the lipids were extruded for 15 cycles through two stacked 0.08 μm polycarbonate membranes using a miniextruder (Avanti). Free Dox and SP were removed by chromatography on Sepharose CL-4B (Sigma). Lipid content in purified liposomes, named Lip(Dox)−SP, was determined by phosphate assay (Pierce, Rockford, IL), Dox content was determined fluorometrically (excitation at 470 nm, emission at 590 nm), and SP content was determined by micro-BCA protein assay (Pierce). The liposome surface Zeta potential and size were measured by Delsa 440SX Zeta Potential Analyzer (Coulter-Beckman, Miami, FL). Complexes of Lip(Dox)−SP with VEGF had an average size of 139 nm (SD = 26) and a zeta-potential of 0.45 mV (SD = 2.25) indicating that our procedure yielded unilamellar liposomes.

The sensitivity of 293 and 293/KDR cells to free Dox was determined using MTT cell proliferation assay (Promega). Briefly, 293 and 293/KDR cells were seeded on 96-well plate, 10^3 cells/well. Twenty-four hours later cells in quadruplicate wells were treated with Dox at a concentration range of 0.1 pM to 10 μM. After 24 h of treatment, the Dox-containing medium was replaced with fresh culture medium. MTT cell proliferation assay was performed according to the manufacturer's instructions after additional 48 h of incubation under the normal culture conditions.

Preparation of Polyethylenimine/S-Protein Conjugates. Linear 25 kDa polyethylenimine (PEI, Polysciences Inc., Warrington, PA) at concentration of 5 mM was modified with 5 mM Traut's reagent (Pierce) in 50 mM triethanolamine, 0.15 M NaCl, 1 mM EDTA, pH 8.0,

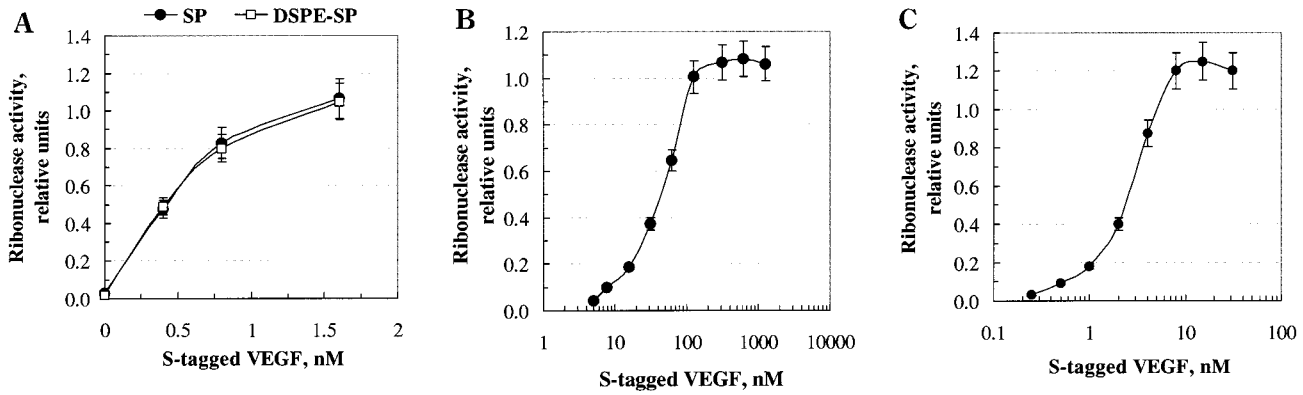

Figure 2. Derivatized S-protein (SP) binds to S-tagged VEGF. Derivatized SP was mixed with varying amounts of S-tagged VEGF. Ribonuclease activity was measured using S-tag Rapid Assay Kit (Novagen). A, DSPE-SP and control SP at concentrations of 4 nM, each. B, Lip(Dox)−SP, at SP concentration of 580 nM. C, PEI−SP, at SP concentration of 6 nM.

under argon at room temperature for 20 min. Concentration of SH groups in modified PEI was determined by Ellman's reagent (Pierce). SP at concentration of 0.66 mM was modified with 1.32 mM sulfo-MBS (Pierce) in conjugation buffer containing 0.1 M NaPi, 0.15 M NaCl, pH 7.2, at room temperature for 30 min. Modified SP and PEI were mixed at the molar ratio of 1:1 in conjugation buffer and incubated at room temperature for 4 h with constant agitation. PEI−SP conjugates were purified by gel-filtration on SE 100/40 (BioRad, Hercules, CA).

Preparation of DNA/PEI−SP−VEGF Complexes. DNA/PEI−SP−VEGF complexes were prepared as described (*5*). The surface zeta potential was measured by a Delsa 440SX Zeta Potential Analyzer (Coulter-Beckman). The ability of all cells to accept and express pGL3 DNA was determined using DNA/PEI complexes at N/P ratio from 5 to 13. The transfectability of VEGFR-2 expressing cells and control cells was found to be similar within 15% experimental error (data not shown).

Measurements of Ribonuclease Activity. Ribonuclease activity was measured with S-tag Rapid Assay Kit (Novagen). A relative unit of ribonuclease activity is 1 OU of acid-soluble material released from polyC incubated with various complexes of S-protein and S-tagged VEGF under the manufacturer's conditions.

RESULTS AND DISCUSSION

Construction of Molecular Drug Delivery Vehicles. The overall design of the modular drug delivery vehicle is shown in Figure 1. In our approach, conjugating the appropriate drug carriers to a universal adapter protein creates standardized payload modules. The role of the adapter protein is to bind with high affinity to a specific peptide fused to a targeting protein. This peptide serves as a "docking tag" for a payload module. In principle, any recombinant targeting protein expressed with such a docking tag can bind a premade adapter−carrier module, and the assembled vehicle can be used for targeted drug delivery.

A number of peptide tags recognized by specific proteins have already been developed for purification and detection of recombinant proteins. Among these, the so-called S-tag system based on interaction between S-peptide and S-protein fragments of bovine ribonuclease A appears to be the best choice for our purposes for the following reasons. First, the 15-aa S-peptide and 104-aa S-protein (SP) fragments form the smallest tag/adapter complex currently available with a K_d of 10^{-9} M^{-1} (*9*). Second, the S-peptide/SP complex is an active ribonuclease (known as RNase S), and therefore association of S-tagged recombinant proteins with SP-carrier modules can be readily detected by the reconstituted ribonuclease activity (*10*). Third, the bovine S-peptide/SP complex has been extensively studied for more than 40 years, detailed information about various aspects of this system is available, and bacterial expression plasmids for production of S-tagged proteins as well as S-protein are commercially available (*9*−*13*). Finally, the complex can be "humanized" by using appropriate fragments of human ribonuclease I (RNase I), thus improving chances for therapeutic success of the proposed strategy.

As a targeting protein we have chosen VEGF, the most specific cytokine for endothelial cells (*14*). The VEGF targeting ability is further narrowed by the evidence that its receptor, VEGFR-2 (KDR/Flk-1), is overexpressed at the sites of pathological angiogenesis (*15*−*17*). This exquisite specificity marks VEGFR-2 as a prominent target for anti-angiogenic therapy. Indeed, several recent reports indicated that VEGF-toxin fusion proteins and anti-VEGFR-2 neutralizing antibodies display highly selective activity in vivo and in vitro (*6*, *18*, *19*). Within VEGF family, $VEGF_{121}$ appears to be the most attractive candidate to be used as a targeting protein for two reasons. First, it has selective affinity to VEGFR-2 and second, it lacks a heparin-binding domain and, therefore, cannot be sequestered in the extracellular matrix. We have expressed $VEGF_{121}$ as a fusion protein containing bovine S-tag as a docking tag (S-tagged VEGF). Using SP as an adapter we have constructed payload modules for liposome delivery and for gene delivery.

Payload Module SP Binds to S-Tagged VEGF. To construct a payload module for liposome delivery, SP was conjugated to distearoyl phosphatidylethanolamine (DSPE) modified with poly(ethylene glycol). Derivatized SP (DSPE−SP) formed catalytically active complexes with S-tagged VEGF as effectively as unmodified SP ($K_d \sim 0.5$ nM), as judged by concentration dependence of ribonuclease activity of these complexes (Figure 2*A*). The fully assembled doxorubicin-loaded, DSPE−SP containing liposomes (Lip(Dox)−SP) also retained the ability to bind S-tagged VEGF (Figure 2B). The enzymatic activity of Lip(Dox)−SP−VEGF complexes was detectable at concentrations 50−100 fold higher than that of DSPE−SP−VEGF (compare Figure 2A and 2B), suggesting lower specific activity of liposome-associated reconstructed ribonuclease. In experiments with Lip(Dox)−SP−VEGF, the total concentration of SP was 580 nM and ribonuclease activity of the complexes reached a plateau at

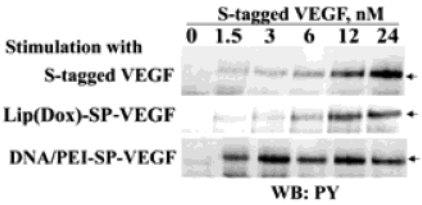

Figure 3. VEGF-based molecular delivery vehicles activate cellular VEGFR-2. Near confluent 293/KDR cells were incubated overnight in DMEM with 0.5% FBS, and then shifted to serum-free DMEM with 0.5 mM sodium vanadate for 5 min at 37 °C. Cells were stimulated with free S-tagged VEGF or VEGF-based delivery vehicles for 5 min at 37 °C, lysed, and analyzed by Western blotting using anti-phosphotyrosine RC20:HRPO conjugate (BD Transduction Labs, San Diego, CA). Arrows indicate positions of VEGFR-2.

~300 nM S-tagged VEGF as expected if SP is randomly distributed between the inner and the outer surfaces of liposomes.

To construct a payload module for gene delivery, SP was conjugated to polyethylenimine (PEI), a well-characterized DNA carrier (*20*−*22*). The conjugation protocol included (i) introduction of SH-group in PEI, (ii) introduction of a maleimide group in SP, and (iii) cross-linking of the modified PEI and SP via a reaction between SH and maleimide groups. We found that SP covalently bound to PEI (PEI−SP) binds S-tagged VEGF with nanomolar (K_d ~2 nM) affinity (Figure 2C).

VEGF Associated with Payload Modules Binds and Activates VEGFR-2. To test the functional activity of S-tagged VEGF in assembled delivery vehicles, we employed a VEGFR-2 tyrosine autophosphorylation assay. As a control we used free S-tagged VEGF. In this assay, assembled doxorubicin-loaded liposome delivery vehicles (Lip(Dox)−SP−VEGF) were as active as S-tagged VEGF alone, suggesting that binding of VEGF to the liposome surface did not affect its interaction with VEGFR-2 (Figure 3).

DNA delivery vehicles (DNA/PEI−SP−VEGF) containing pGL3 DNA at the N/P ratio of 8.4 (where N is the number of amino groups in PEI and P is the number of DNA phosphate groups) induced VEGFR-2 tyrosine autophosphorylation at VEGF concentrations as low as 1.5 nM (Figure 3). Importantly, DNA/PEI−SP−VEGF complexes were more efficient in this assay than free S-tagged VEGF. This effect can be explained by the higher avidity of DNA/PEI−SP−VEGF complexes to cells overexpressing VEGFR-2. Indeed, at the N/P ratio of 8.4 every pGL3 plasmid (5256 bp) is bound to several 25 kDa PEI (~600 monomers per molecule), and the complexes are packaged into highly condensed particles (*20*). Since every PEI−SP carries VEGF, assembled DNA/PEI−SP−VEGF will contain several VEGF molecules per a tightly packed complex and therefore can bind to cellular VEGFR-2 with higher avidity than individual VEGF.

VEGF-Based Targeted Delivery of Therapeutics. Lip(Dox)−SP−VEGF were tested on 293/KDR (2.5×10^6 VEGFR-2/cell) and 293 parental cells. To account for nonreceptor mediated toxicity, 293/KDR and 293 were treated with Lip(Dox)−SP and growth inhibition induced by Lip(Dox)−SP−VEGF was calculated relative to these controls. There were no difference in sensitivity of 293/KDR and 293 cells to Lip(Dox)−SP (data not shown). We found that Lip(Dox)−SP−VEGF did not affect 293, but inhibited growth of 293/KDR in a dose-dependent manner (Figure 4*A*) with IC_{50} of 0.8 nM for equivalent concentration of Dox. 293/KDR and 293 displayed similar sensitivity to free Dox with IC_{50} of 8 nM and 10 nM, respectively

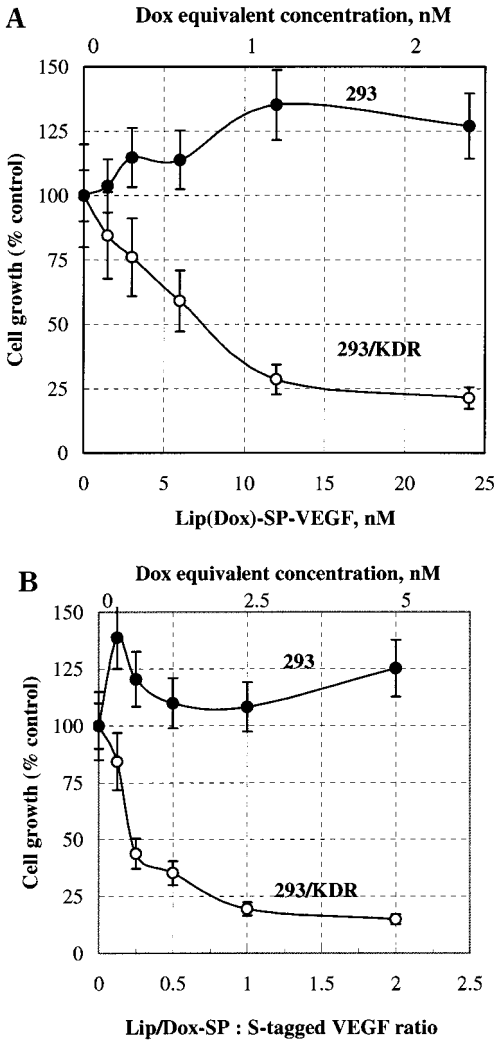

Figure 4. VEGF targets liposomes to cells overexpressing VEGFR-2. 293/KDR and 293 parental cells were plated on 24-well plates, 5×10^3 cells/well 20 h before the experiment. Lip(Dox)−SP were mixed with S-tagged VEGF at varying SP to VEGF molar ratios in serum-free DMEM, incubated on ice for 20 min, and then added to cells in complete culture medium in triplicate wells. After 20 h, cells were shifted to fresh culture medium. Cells were counted in a Coulter Counter (Coulter-Beckman) after 3-day incubations. Control cells were treated with equal amounts of Lip(Dox)−SP without VEGF and growth inhibition induced by Lip(Dox)−SP−VEGF was calculated relative to these controls. Liposome delivery experiments were repeated two times. A, Lip(Dox)−SP−VEGF at liposome SP to VEGF ratio of 1:1. Dox equivalent concentration is indicated. B, Varying amounts of Lip(Dox)−SP and constant 12 nM VEGF.

(data not shown). Thus, a 10-fold decrease in IC_{50} for 293/KDR can be attributed to VEGF-mediated delivery of liposome-encapsulated Dox. Similar decrease in IC_{50} was reported for human umbilical vein endothelial cells (HUVEC) treated with immunoliposome-encapsulated and free Dox (23). Interestingly, effect Lip(Dox)−SP−VEGF on 293/KDR was close to saturation at the liposome SP to S-tagged VEGF monomer ratio of 0.5:1 (Figure 4*B*). The latter result indicated that docking of Lip(Dox)−SP to both S-tags in the VEGF dimer does not significantly improve interaction of the resulting complex with cellular VEGFR-2.

VEGF-based DNA delivery vehicles were tested on three cell lines expressed varying number of VEGFR-2 per cell: PAE/0.1-KDR (2.5×10^4 VEGFR-2/cell), PAE/KDR (2.5×10^5 VEGFR-2/cell), and 293/KDR. PAE/V and 293/V cells transfected with empty vectors served as

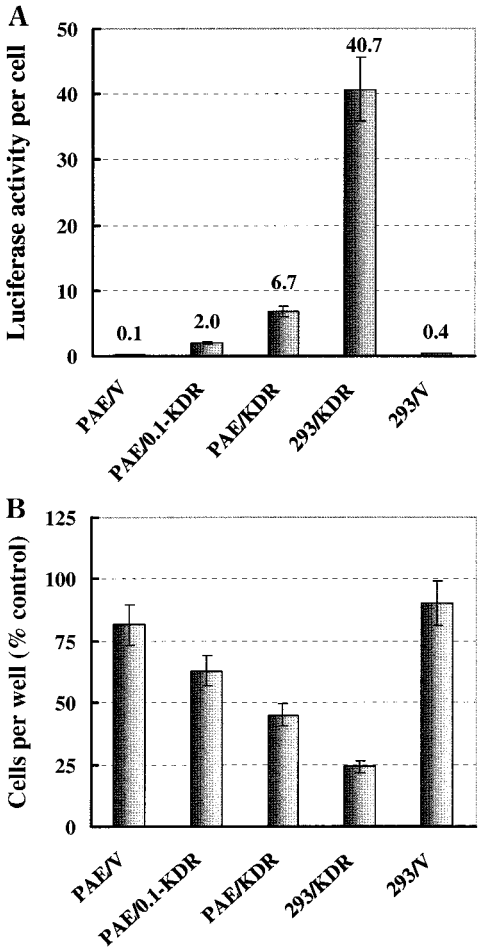

Figure 5. VEGF targets DNA delivery vehicles to cells overexpressing VEGFR-2. Cells were plated on 24-well plates at densities of 4×10^3 cells/well for PAE, PAE/KDR, PAE/0.1-KDR, and 2×10^4 cells/well for 293 and 293/KDR, 20 h before the experiment. PEI−SP was mixed with S-tagged VEGF (SP to VEGF ratio of 1:1.5) in 5% glucose, incubated on ice for 20 min, added dropwise to DNA in 5% glucose (N/P of 8.4), incubated on ice for an additional 20 min, and added to cells in complete culture medium to final concentrations of 12 nM VEGF, 36 nM PEI−SP, and 0.7 μg DNA per well. DNA delivery experiments were repeated three times. A, 40 h after luciferase DNA delivery, cells from triplicate wells were lyzed and assayed for luciferase activity, while cells from duplicate wells were washed with PBS, trypsinized, and counted in a Coulter Counter. B, 70 h after caspase DNA delivery, cells from triplicate wells were washed with PBS, trypsinized, and counted in a Coulter Counter.

controls. To reduce nonspecific DNA delivery, the DNA/PEI−SP−VEGF complexes were assembled with an N/P ratio of 8.4 giving a negative surface charge (zeta-potential of −25.4 mV, SD = 9.4). The efficiency of luciferase DNA delivery to cells depended on the cellular density of VEGFR-2, with the highest luciferase activity detected in 293/KDR cells expressing ~2.5×10^6 VEGFR-2/cell (Figure 5*A*). It should be noted that we have recently found that freshly isolated human circulating endothelial precursor cells express more VEGFR-2 than 293/KDR, indicating that VEGFR-2 may be overexpressed to very high density on certain cells in vivo (manuscript in preparation).

To test whether the VEGF-based vehicle can deliver therapeutically significant quantities of DNA to targeted cells, we have loaded the vehicle with the plasmid expressing human pro-caspase 8b, an apical protease of the apoptotic cascade (*24*). We found that VEGF-based caspase DNA delivery resulted in preferential death of cells expressing VEGFR-2 and that this effect also depended on the level of VEGFR-2 per cell (Figure 5B). Thus, we can expect that VEGF-based delivery vehicles in vivo will more efficiently target cells overexpressing VEGFR-2, such as endothelial cells at the sites of pathological angiogenesis or endothelial progenitor cells.

Our results provide evidence that VEGF-based molecular delivery vehicles can be constructed without random chemical modification of the targeting protein. We believe that molecular vehicles for delivery of therapeutic and imaging agents may be constructed using other targeting proteins, different drug carriers, and new payload modules. We expect that construction of a targeting protein, which can be combined with premade payload modules, would require significantly less effort than development of a customized chemical modification procedure for the same protein or development of a heterobifunctional antibody against the same target. Our recent experiments indicate that adapter/docking tag system can be based on human RNase I, and that random chemical modification of the adapter protein can be avoided by engineering an additional cysteine residue into S-protein fragment of RNase I (manuscript in preparation). Experiments are now in progress to develop novel payload modules based on humanized adapter/docking tag system.

ACKNOWLEDGMENT

This work was supported in part by a grant from National Institutes of Health (# 2 R44 HL61143-02). We thank C. Hamby for critical reading of the manuscript and A. Backer for help with graphic.

LITERATURE CITED

(1) Dubowchik, G. M., and Walker, M. A. (1999) Receptor-mediated and enzyme-dependent targeting of cytotoxic anticancer drugs. *Pharmacol. Ther. 83*, 67−123.

(2) Wilbur, D. S., Pathare, P. M., Hamlin, D. K., Stayton, P. S., To, R., Klumb, L. A., Buhler, K. R., and Vessella, R. L. (1999) Development of new biotin/streptavidin reagents for pretargeting. *Biomol. Eng. 16*, 113−118.

(3) Kriangkum, J., Xu, B.. Nagata, L. P., Fulton, R. E., and Suresh, M. R. (2001) Bispecific and bifunctional single chain recombinant antibodies. *Biomol. Eng. 18*, 31−40.

(4) Zhu, H., Jain, R. K., and Baxter, L. T. (1998) Tumor pretargeting for radioimmunodetection and radioimmunotherapy. *J. Nuclear Med. 39*, 65−76.

(5) Gaidamakova, E. K., Backer, M. V., and Backer, J. M. (2001) Molecular vehicle for target-mediated delivery of therapeutics and diagnostics. *J. Controlled Release 74*, 341−347.

(6) Backer, M. V., and Backer, J. M (2001) Targeting endothelial cells overexpressing VEGFR-2: selective toxicity of Shiga-like toxin-VEGF fusion proteins. *Bioconjugate Chem. 12*, 1066−1073.

(7) Backer, M. V., and Backer, J. M. (2001) Functionally active VEGF fusion proteins. *Prot. Expr., Purif. 23*, 1−7.

(8) Li, B., Fuh, G., Meng, G., Xin, X., Gerritsen, M. E., Cunningham, B., and de Vos A. M. (2000). Receptor-selective variants of human vascular endothelial growth factor. *J. Biol. Chem. 275*, 29823−29828.

(9) Kim, J. S., and Raines, R. T. (1993) Ribonuclease S-peptide as a carrier in fusion proteins. *Protein Sci. 2*, 348−356.

(10) Richards, F. M., and Wyskoff, H. W. (1971) in *The Enzymes* (P. D. Boyer, Ed.) pp 647−806, Academic Press, New York.

(11) Chakshusmathi, G., Girish, S., Ratnaparkhi, S., Madhu, P. K., and Varadarajan, R. (1999) Native-state hydrogen-exchange studies of a fragment complex can provide structural information about the isolated fragments. *Proc. Natl. Acad. Sci. U. S. A. 96*, 7899−7904.

(12) Connelly, P. R., Varadarajan, R., Sturtevant, J. M., and Richards, F. M (1990) Thermodynamics of protein-peptide interactions in the ribonuclease S system studied by titration calorimetry. *Biochemistry 29*, 6108−6114.

(13) Kim, E. E., Varadarajan, R., Wyckoff, H. W., and Richards, F. M. (1992) Refinement of the crystal structure of ribonuclease S. Comparison with and between the various ribonuclease A structures. *Biochemistry 31*, 12304−12314.

(14) Senger, D. R., Van de Water, L., Brown, L. F., Nagy, J. A., Yeo, K. T., Yeo, T. K., Berse, B., Jackman, R. W., Dvorak, A. M., and Dvorak, H. F. (1993) Vascular permeability factor (VPF, VEGF) in tumor biology. *Cancer Metastasis Rev. 12*, 303−324.

(15) Feng, D., Nagy, J. A., Brekken, R. A., Pettersson, A., Manseau, E. J., Pyne, K., Mulligan, R., Thorpe, P. E., Dvorak, H. F., and Dvorak, A. M. (2000) Ultrastructural localization of the vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) receptor-2 (FLK-1, KDR) in normal mouse kidney and in the hyperpermeable vessels induced by VPF/VEGF-expressing tumors and adenoviral vectors. *J. Histochem. Cytochem. 48*, 545−556.

(16) Brown, L., Detmar, M., Claffey, K., Nagy, J., Feng, D., Dvorak, A. M., and Dvorak, H. F. (1997) Vascular permeability factor/vascular endothelial growth factor: a multifunctional angiogenic cytokine, in *Regulation of Angiogenesis* (I. Goldberg, E. Rosen, Eds) pp 233−269, Basel, Birkhouser Verlag.

(17) Detmar, M., Brown, L. F., Claffey, K. P., Yeo, K. T., Kocher, O., Jackman, R. W., Berse, B., and Dvorak, H. F. (1994) Overexpression of vascular permeability factor/vascular endothelial growth factor and its receptors in psoriasis. *J. Exp. Med. 180*, 1141−1146.

(18) Arora, N., Maood, R., Zheng, T., Cai, J., Smith, L., and Gill, P. S. (1999) Vascular endothelial growth factor chimeric toxin is highly active against endothelial cells. *Cancer Res. 59*, 183−188.

(19) Prewett, M., Huber, J., Li, Y., Santiago, A., O'Connor, W., King, K., Overholser, J., Hooper, A., Pytowski, B., Witte, L., Bohlen, P., and Hicklin, D. J. (1999) Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody inhibits tumor angiogenesis and growth of several mouse and human tumors. *Cancer Res. 59*, 5209−5218.

(20) Boussif, O., Lezoualch, F., Zanta, M.-A.,. Mergny, M. D, Scherman, D., Demeneix, B., and Behr, J.-P. (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo. *Proc. Natl. Acad. Sci. U. S. A. 92*, 7297−7301.

(21) Zanta, M.-A., Boussif, O., Adib, A., and Behr, J.-P. (1997) In vitro gene delivery to hepatocytes with galactosylated polyethylenimine. *Bioconjugate Chem. 8*, 839−844.

(22) Rolland, A. (1998) From genes to gene medicines: recent advances in nonviral gene delivery. *Crit. Rev. Ther. Drug Carrier Syst. 15*, 143−198.

(23) Spragg, D. D., Alford, D. R., Greferath, R., Larsen, C. E., Lee, K.-D., Gurtner, G. C., Cybulsky, M. I., Tosi P. F., Nicolau, C., and Gimbrone, M. A., Jr. (1997) Immunotargeting of liposomes to activated vascular endothelial cells: A strategy for site-selective delivery in the cardiovascular system. *Proc. Natl. Acad. Sci. U. S. A. 94*, 8795−8800.

(24) Cohen, G. M. (1997) Caspases: the executioners of apoptosis. *Biochem. J. 326*, 1−16.

BC0155770