Brief Articles

Lipopeptide with a RGDK Tetrapeptide Sequence Can Selectively Target Genes to Proangiogenic α5β1 Integrin Receptor and Mouse Tumor Vasculature

Dipankar Pramanik,[†,‡] Bharat K. Majeti,[†,⊥,‡] Goutam Mondal,[‡] Priya P. Karmali,[#,‡] Ramakrishna Sistla,[§] Obula G. Ramprasad,[||] Gunda Srinivas,[||] Gopal Pande,[||] and Arabinda Chaudhuri*[,‡]

Division of Lipid Science and Technology, Pharmacology Division, Indian Institute of Chemical Technology, Hyderabad 500607, India, Centre for Cellular and Molecular Biology, Hyderabad 500607, India

Received July 23, 2008

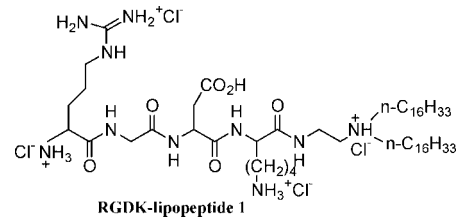
Integrins, the major class of αβ heterodimeric transmembrane glycoprotein receptors, play crucial roles in mediating tumor angiogenesis. Genetic ablation experiments combined with use of antibodies/peptide ligands for blocking either α_5 or β_1 integrins have convincingly demonstrated $\alpha_5\beta_1$ integrin to be unquestionably proangiogenic among the 24 known integrin receptors. Herein, we report on a novel RGDK-lipopeptide **1** that targets selectively $\alpha_5\beta_1$ integrin and is capable of targeting genes to mouse tumor vasculatures.

Introduction

Angiogenesis, the sprouting of new blood vessels from preexisting vessels, is a remarkable feature of tumor growth.[1] Prevention of new blood vessel formation around tumor tissues is a promising antiangiogenic therapeutic approach to combat cancers. In such antiangiogenic cancer therapy, selective targeting of anticancer drugs/genes to tumor vasculatures is accomplished by exploiting unique molecular markers overexpressed in the tumor endothelial cells. Integrins, the major class of αβ heterodimeric transmembrane glycoprotein receptors, belong to one such unique class of molecular markers. Among the total 24 integrins, αvβ3, αvβ5, and α5β1 integrins have received extensive exploitations as antiangiogenic drug targets. Integrins mediate cellular adhesion to ECM or to adjacent cells and can regulate cell survival and proliferation by modulating intracellular signaling processes.[2−6] Because many integrins and their specific extracellular matrix ligands play crucial roles in mediating tumor angiogenesis, they are gaining increasing importance as drug targets in antiangiogenic cancer therapy.[7]

Brooks et al. and others reported that the monoclonal antibody (LM609) as well as the various RGD-tripeptide based low-molecular-weight reagents are recognized by the integrins αvβ3 and αvβ5 and these reagents are capable of blocking tumor and retinal angiogenesis in response to growth factors.[8−10] However, the findings in a series of gene ablation experiments involving the use of αv or β3 and/or β5 deficient mice have seriously questioned the proangiogenic nature of these two integrins. Mice lacking αv or β3 and/or β5 deficient genes have been shown to be viable and fertile.[11,12] Perhaps the proangiogenic nature of the αvβ3 and αvβ5 integrins has been most seriously challenged by the findings that mice lacking αv integrins exhibit extensive tumor growth and angiogenesis.[13] Importantly, no such discrepancy between the genetic results and those obtained using antibodies or low-molecular weight reagents exist for the integrin α5β1 and its ligands. Use of antibodies/peptide ligands for blocking either α5 or β1 integrins inhibited angiogenesis.[14] Genetic ablation of either α5 or β1 integrins in mice led to embryonic lethality with major vascular defects.[15,16] Thus, gene ablation experiments combined with the use of antibodies/peptide ligands have convincingly demonstrated α5β1 integrin as an unquestionably proangiogenic integrin receptor. Ever since these reports on the distinct proangiogenic roles of the α5β1 integrins were disclosed, efforts toward developing efficient α5β1 integrin receptor ligands and antagonists targeting systems are gaining increasing importance. Reports on the developments of efficient and selective nonpeptidic ligands and antagonists of α5β1 integrin receptor have been published.[17−20] Zimmermann et al. reported their in vitro studies on the development of two cyclic penta- and hexapeptide ligands containing RGD sequence and a β-amino acid with considerable affinity for α5β1 integrin receptor.[21] Herein, we show that lipopeptide containing a lysine functionality immediately after the RGD sequence (RGDK-lipopeptide **1**, Scheme 1) in its polar headgroup region can selectively target genes to α5β1 integrin receptors. When the lysine residue of the RGDK tetrapeptide sequence was replaced with a leucine residue, the resulting RGDL-lipopeptide **3** did not show any α5β1 integrin receptors selective transfection

Scheme 1. Structure of the α5β1 integrin receptor and tumor vasculature targeting RGDK-lipopeptide **1**



* To whom correspondence should be addressed. Phone: 91 40 27193201. Fax: 91 40 27193370. E-mail: arabinda@iict.res.in.
† Equally contributing authors.
‡ Division of Lipid Science and Technology, Indian Institute of Chemical Technology.
§ Pharmacology Division, Indian Institute of Chemical Technology.
|| Centre for Cellular and Molecular Biology.
⊥ Present address: Laboratory of David A. Cheresh, Department of Pathology, University of California, San Diego, School of Medicine & Moores UCSD Cancer Center, 3855 Health Sciences Drive 0803, La Jolla, CA 92093-0803.
Present address: Laboratory of Erkki Ruoslahti, Cancer Research Center, The Burnham Institute, La Jolla, CA 92037.

10.1021/jm800915y CCC: $40.75
Published on Web 10/29/2008

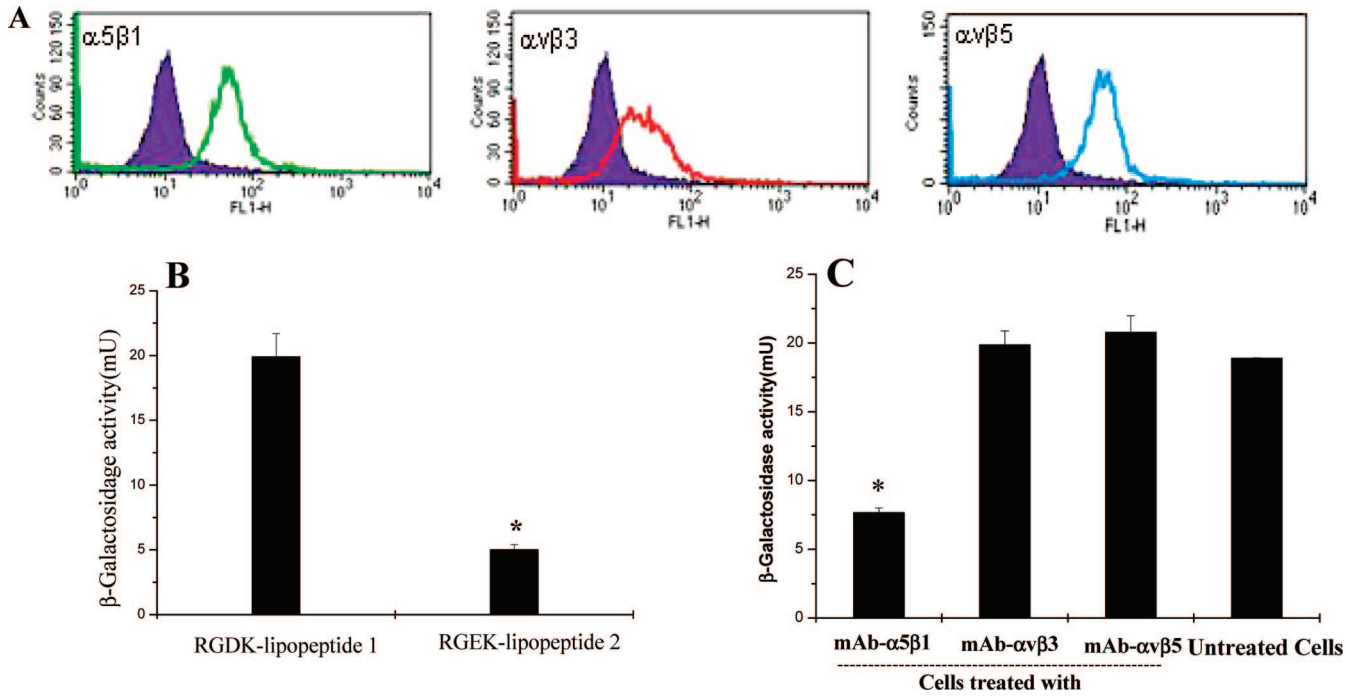


Figure 1. RGDK-lipopeptide **1** targets genes selectively to α5β1 integrin receptors. (A) Evaluation of the α5β1, αvβ3, and αvβ5 integrins in A549 cells by FACS. In each of the three experiments, about one million cells were fixed and labeled with monoclonal antibodies against α5β1, αvβ3, and αvβ5 integrins and then examined by flow cytometry. The blue shaded areas on the left side in each FACS profile represents the signal of the control isotype antibody, and the traces on the right represent the signal with anti-α5β1 (green), antiαvβ3 (red), and antiαvβ5 (indigo) integrin antibodies. (B) Relative efficacies of the RGDK-lipopeptide **1** and the control RGEK-lipopeptide **2** in transfecting A549 cells (*P < 0.0005). (C) Gene transfer efficiencies of the RGDK-lipopeptide **1** in A549 cells (measured using the lipofection method described in ref 32) in the presence of monoclonal antibodies against α5β1, αvβ3, and αvβ5 integrins (*P < 0.005 when compared to the control transfection experiments in absence of any added antibody).

properties. This demonstrates that the lysine functionality in RGDK-lipopeptide **1** plays a crucial role behind its α5β1 integrin receptor specificity. Immunohistochemical findings demonstrate that the RGDK-lipopeptide **1** is capable of delivering genes to the mouse tumor vasculatures under systemic settings. Importantly, remarkable inhibition of tumor growth was observed when the electrostatic complex of the RGDK-lipopeptide **1** and the anticancer p53 gene was intravenously administered in C57BL/6 mice bearing an aggressive B16F1 tumor.

Results and Discussion

The amino acid sequence arginine-glycine-aspartic acid (RGD) is the most evolutionary conserved feature of many natural integrin-binding extracellular matrix proteins, and integrin receptors potentiate cellular internalization processes for many viruses.[22−24] On the basis of this rationale, efficient integrin receptor targeting systems consisting of RGD-modified liposomes,[25−27] cRGD-functionalized polymeric micelles,[28] and lipid based nanoparticles containing a covalently grafted integrin ligands[29,30] have been reported. However, most of these prior reported lipopeptide systems containing the RGD sequence are αvβ3 integrin receptor specific and none of these RGD-lipopeptide systems are capable of selectively targeting the unquestionably proangiogenic α5β1 integrin receptors. Herein, we demonstrate for the first time that a cationic lipopeptide containing a RGDK tetrapeptide sequence in its polar headgroup region can selectively target genes to the proangiogenic α5β1 integrin receptors. The RGDK-lipopeptide **1** was synthesized by covalently grafting the RGD-tripeptide sequence to the lysine residue of a previously described monolysinylated cationic amphiphile.[31] The purities of all the final lipopeptides were confirmed by reversed phase analytical HPLC (Figures S1−S3, Supporting Information). Details of syntheses, purifications, ^{1}H NMR, and high-resolution mass spectral characterizations for the RGDK-lipopeptide **1** and the control lipopeptides, namely RGEK-lipopeptide **2** and RGDL-lipeptide **3**, are provided in the Supporting Information.

To begin with, we tested the efficiencies of the RGDK-lipopeptide **1** in delivering genes into A549 cells (human lung carcinoma cells) following previously described liposomal gene delivery protocol.[32] Briefly, electrostatic complex of a plasmid DNA (pCMV-SPORT-β-Gal reporter gene encoding the enzyme β-galactosidase) and the cationic liposomes of the lipopeptides (containing 2:1 mole ratio of RGDK-lipopeptide **1** and Chol[a]) were incubated with the cells. After 48 h (which ensured transgene expression in the transfected cells), cells were lysed and the activities of the expressed β-galactosidase were assayed by adding a spectrophotometric substrate of β-galactosidase (*ortho*-nitrophenyl-β-galactoside). Fluorescence-assisted cell sorting (FACS) analysis revealed the presence of all the three αvβ3, αvβ5, and α5β1 integrin receptors expressed on its surface (Figure 1A). As depicted in Figure 1B, the RGDK-lipopeptide **1** was found to be efficient in transfecting A549 cells. RGD and not RGE is the ligand for integrin receptors.[33] With a view to confirm the involvement of integrin receptor in the cellular uptake of RGDK-lipopeptide **1**:p-CMV-SPORT-β-Gal gene complex, we synthesized the control RGEK-lipopeptide **2** and evaluated its gene transfer property in A549 cells. The gene transfer efficacy of the control RGEK-lipopeptide **2** was found to be remarkably compromised compared to that of

[a] Abbreviations: Chol: cholesterol; DCM: dichloromethane; DMEM: Dulbecco's modified Eagles medium; FBS: fetal bovine serum; ONPG: *o*-nitrophenyl-β-D-galactopyranoside; PBS: phosphate buffered saline.

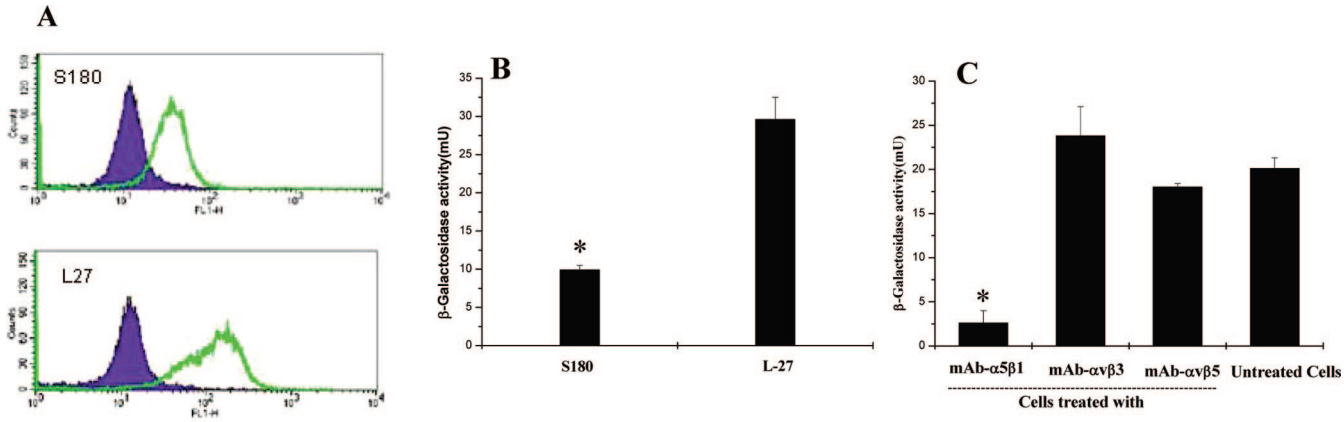


Figure 2. Enhanced transfection efficiencies of the RGDK-lipopeptide **1** in L27 cells with overexpressed $\alpha5\beta1$ integrins. (A) FACS profiles of the $\alpha5\beta1$ integrins in S180 and L27 (S180 cells transformed with human α5 cDNA) cells. (B) Enhanced gene transfer efficiencies of the RGDK-lipopeptide **1** in L27 cells compared to that in S180 cells ($*P < 0.001$). (C) Gene transfer efficiencies of the RGDK-lipopeptide **1** in L27 cells pretreated with monoclonal antibodies against $\alpha5\beta1$, $\alpha v\beta3$, and $\alpha v\beta5$ integrins ($*P < 0.001$ compared to transfection efficacies in untreated cells).

the RGDK-lipopeptide **1** (Figure 1B). Such significantly reduced gene delivery efficiency of the control RGEK-lipopeptide **2** (compared to that of RGDK-lipopeptide **1**) supports the notion that the cellular uptake of the RGDK-lipopeptide **1**:DNA complexes is mediated via integrin receptors

Next, with a view to gaining insights into whether the RGDK-lipopeptide **1** is capable of delivering genes into cells via any specific integrin receptor among the $\alpha v\beta3$, $\alpha v\beta5$, and $\alpha5\beta1$ integrin receptors, we measured the gene transfer efficiencies of the RGDK-lipopeptide **1** in A549 cells preincubated with the monoclonal antibodies against these three integrins. The transfection efficiencies of the RGDK-lipopeptide **1** were found to be minimally affected when A549 cells were preincubated with monoclonal antibodies against $\alpha v\beta3$ and $\alpha v\beta5$ integrins (Figure 1C). Thus, the cellular uptake of the lipopeptide:DNA complexes is unlikely to be mediated via either a $\alpha v\beta3$ or a $\alpha v\beta5$ integrin receptor. Contrastingly, the RGDK-lipopeptide **1** was found to be significantly less efficient in delivering genes (by ~60%) when A549 cells were preincubated with the monoclonal anti-$\alpha5\beta1$ integrin antibodies (Figure 1C). Thus, the findings summarized in Figure 1C provided convincing evidence for the $\alpha5\beta1$ integrin receptor specific transfection properties of the RGDK-lipopeptide **1**. The $\alpha5\beta1$ integrin selectivity of the RGDK-lipopeptide **1** was further confirmed by conducting the transfection experiments in S180 (murine sarcoma cells) and L27 cells (S180 cells stably transfected with human α5).[34] That the degree of $\alpha5\beta1$ integrin receptors expression in L27 cell surface is significantly higher than that in S180 cells was confirmed by the findings in the conventional FACS analysis (Figure 2A). Consistent with the $\alpha5\beta1$ integrin receptor selectivity of the RGDK-lipopeptide **1** and the higher amount of $\alpha5\beta1$ integrin receptor expression in L27 cell surface (compared to that in S180 cells), the RGDK-lipopeptide **1** was found to be significantly more efficacious in transfecting L27 cells than in transfecting S180 cells (Figure 2B). Importantly, the transfection efficiencies of the RGDK-lipopeptide **1** were significantly reduced when L27 cells were preincubated with monoclonal anti-$\alpha5\beta1$ integrin antibodies and not with monoclonal antibodies raised against the integrins $\alpha v\beta3$ and $\alpha v\beta5$ (Figure 2C).

We envisaged that the lysine group might be playing an important role behind the above-mentioned $\alpha5\beta1$ integrin receptor specific gene delivery efficiencies of the RGDK-lipopeptide **1**. To address this issue, we synthesized a second

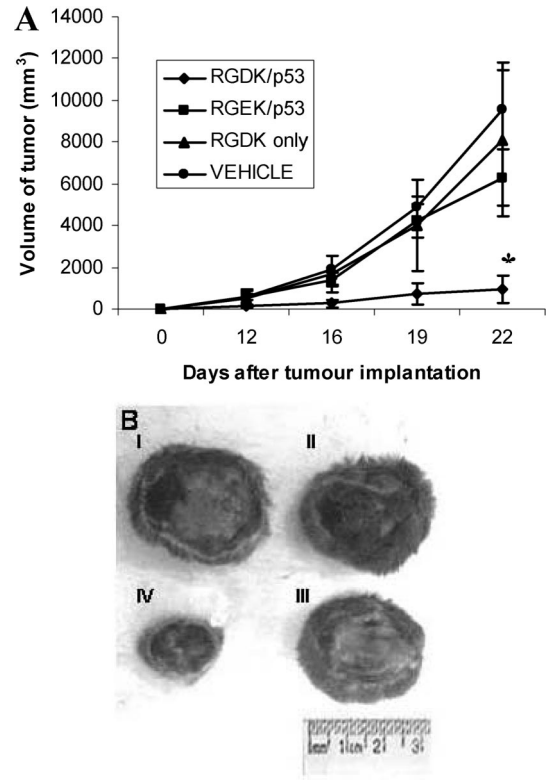


Figure 3. Tumor growth inhibition and tumor vasculature targeting properties of the RGDK-lipopeptide **1**. (A) Six−eight week old female C57BL/6 mice (each weighing 20−22 g) with aggressive B16F1 tumors (produced by subcutaneous injections of 1×10^5 B16F1 cells in 100 μL of Hank's buffer salt solution (HBSS) into the left flanks on day 0) were randomly sorted into four groups and each group ($n = 5$) was administered intravenously with: RGDK-lipopeptide **1**:p53 lipoplex in 5% aqueous glucose (filled diamonds), RGEK-lipopeptide **2**:p53 lipoplex in 5% aqueous glucose (filled squares), liposomes of RGDK-lipopeptide **1** alone (filled triangles) and 5% aqueous glucose alone (filled circles) on days 5, 7, 9, 11, and 14. Tumor volumes ($V = {}^1/_2 \cdot ab^2$, where a = maximum length of the tumor and b = minimum length of the tumor measured perpendicular to each other) were measured with a slide calipers for up to 22 days. Results represent the means ± SD ($n = 5$, $*P < 0.005$ compared to RGEK/p53). (B) Representative samples of B16F1 tumors excised on day 22. (I) Tumor treated with vehicle only. (II) Tumors treated with RGDK-liposomes only. (III) Tumors treated with RGEK-liposomes/p53 complexes. (IV) Tumors treated with RGDK-liposomes/p53 complexes.

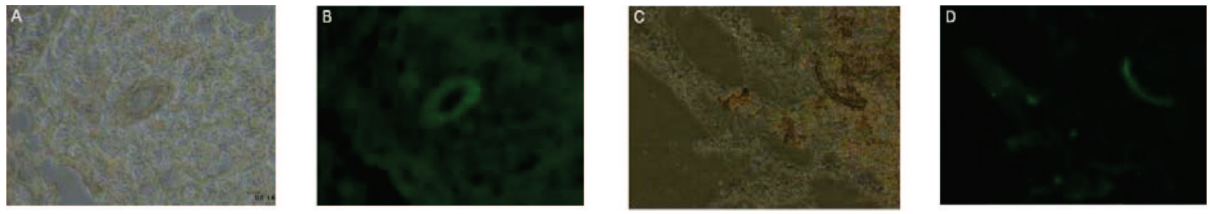


Figure 4. (A,C). Immunohistochemical staining of tumor endothelial cells. On day 22 after tumor inoculation, mice were intravenously injected with the complex of RGDK-lipopeptide **1** and α5-GFP plasmid DNA. After 24 h, tumors were excised, sectioned, and the representative tumor sections were immunostained for tumor endothelial cells with anti-vWF antibodies. (B,D) RGDK-lipopeptide **1** targets genes to tumor neovasculature. The stained tumor slides shown in parts (A) and (C) when observed in the same positions under fluorescent microscope (at 400 magnification) using a green filter revealed GFP expression in the tumor vasculatures. Bar = 1 μm.

control RGDL-lipopeptide **3** following the same peptide coupling strategy as adopted for synthesis of the RGDK-lipopeptide **1** in which the lysine residue was substituted with leucine. Unlike in the case of the RGDK-lipopeptide **1**, the transfection efficiencies of the control RGDL-lipopeptide **3** in A549 cells remained essentially unaffected when the cells were preincubated with monoclonal antibodies against all the three integrins namely, α5β1, α$v\beta$3, and α$v\beta$5 integrins (Figure S4, Supporting Information). Clearly, the control RGDL-lipopeptide **3** does not show any specificity toward α$v\beta$3, α$v\beta$5, and α5β1 integrin receptors. This finding strongly supports the notion that the lysine (K) residue plays a crucial role in imparting selectively α5β1 integrin receptor targeting efficacy to the RGDK-lipopeptide **1**. Future binding studies using purified α5β1 integrin receptors will provide more quantitative information on the relative binding affinities of RGDK- lipopeptide **1** and RGDL-lipopeptide **3** for α5β1 integrin receptor.

Next, with a view to address the systemic potential of the α5β1 integrin receptor specific RGDK-lipopeptide **1**, anticancer p-CMV-p53 gene in complexation with RGDK-lipopeptide **1** was intravenously administered in C57BL/6 mice bearing the aggressive B16F1 tumors (the sizes of the RGDK-lipopeptide **1**:p53 lipoplexes varied within 300−400 nm as measured by dynamic laser light scattering instrument, Malvern, UK). As depicted in Figure 3 (parts A and B), remarkable tumor growth inhibition was observed. The degree of the tumor growth inhibition was low when p-CMV-p53 gene was administered in complexation with the control RGEK-lipopeptide **2**. Because RGD and not RGE is a integrin receptor ligand, this finding is consistent with the involvement of integrin receptor mediated cellular uptake of the RGDK-lipopeptide:p53 complex in the systemic setting too. No significant inhibition of tumor growth was observed when naked p53 plasmid alone (i.e., not in complexation with the RGDK-lipopeptide **1**) were intravenously administered (data not shown), presumably due to its susceptibility to serum nucleases. Mice intravenously administered with vehicle alone (5% aqueous glucose solution) developed large tumors on day 22 (Figure 3, parts A and B) and were sacrificed at that point. Importantly, extent of tumor growth inhibition was much less pronounced for mice intravenously injected with the control RGDL-lipopeptide **3**:p53 complexes (Figure S5, Supporting Information).

Finally, toward probing whether the presently described RGDK-lipopeptide **1** is capable of targeting tumor vasculatures under systemic settings, aggressive B16F1 tumors (murine melanoma tumor) were produced in 6−8 weeks old female C57BL/6 mice (each weighing 20−22 g) by subcutaneous injections of 1×10^5 B16F1 cells in 100 μL of Hank's buffer salt solution (HBSS) into their left flanks. When large tumors were grown (on day 22 after tumor cell inoculation), mice were administered with the electrostatic complexes of RGDK-lipopeptide **1**:p-α5GFP (plasmid DNA encoding green fluorescence protein) via tail-vein injection.

Fixed tumor sections upon immunohistochemical staining with blood vessel marker (anti-vWF antibodies) revealed the presence of the tumor vasculatures when observed in bright field (Figure 4A,C) immunohistochemically stained tumor sections when observed under a fluorescent microscope revealed GFP expression (green fluorescence) in the same tumor vasculatures (Figure 4B,D). Thus, the findings summarized in Figure 4 (parts A−D) convincingly demonstrated the ability of the RGDK-lipopeptide **1** in delivering genes to tumor vasculatures.

Conclusions

Clinical success of antiangiogenic cancer therapy critically depends upon selective delivery of cytotoxic genes/drugs to the endothelial cells of the sprouting new blood vessels around the tumors. Potential targets include mediators of angiogenesis and protein molecules overexpressed in the endothelial cells of tumor neovasculatures. Integrins, the $\alpha\beta$ heterodimeric transmembrane glycoprotein receptors, belong to such a class of molecules that play crucial roles in mediating tumor angiogenesis. Gene ablation experiments combined with use of antibodies/peptide ligands have convincingly demonstrated $\alpha_5\beta_1$ integrin to be the most proangiogenic among these three integrin receptors. Herein, we demonstrate for the first time that lipopeptide containing a lysine functionality after the RGD sequence in its polar headgroup region (RGDK-lipopeptide **1**) can selectively target genes to the proangiogenic α5β1 integrin receptors. When the lysine residue of the RGDK tetrapeptide sequence was replaced with a leucine residue, the resulting RGDL-lipopeptide **3** did not show any α5β1 integrin receptor selective transfection properties. This demonstrates that the lysine functionality in RGDK-lipopeptide **1** plays a crucial role behinds its α5β1 integrin receptor specificity. Immunohistochemical staining of mice tumor sections revealed efficacy of RGDK-lipopeptide **1** in delivering genes to tumor vasculatures. Importantly, remarkable inhibition of tumor growth was observed when the electrostatic complex of the RGDK-lipopeptide **1** and the anticancer p53 gene was intravenously administered in C57BL/6 mice bearing aggressive B16F1 tumor. Availability of the presently described RGDK-lipopeptide **1** is expected to find future clinical exploitation in antiangiogenic cancer therapy including noninvasive imaging of tumor vasculatures.

Acknowledgment. This work was supported by the Department of Biotechnology, Government of India, New Delhi, and the Council of Scientific and Industrial Research, Government of India, New Delhi (NWP 0036). We thank Erkki Ruoslahti for suggesting to us in vivo experiments on C57BL/6 mice bearing aggressive B16F1 tumors, Robert. J. Debs and Nalam. M. Rao for providing the p-CMV-p53 and p-CMV-SPORT-β-

Gal plasmids, respectively, and Dr. Rajkumar Banerjee for his assistance in tumor growth inhibition experiments. D.P., B.K.M., and G.M. thank the Council of Scientific and Industrial Research, Government of India, New Delhi, and P.P.K. thanks the University Grant Commission, Government of India, New Delhi, for providing doctoral research fellowships.

Supporting Information Available: Details of syntheses, purifications, ^{1}H NMR, and high-resolution mass spectral characterizations for the RGDK-lipopeptide **1** and the control lipopeptides, namely RGEK-lipopeptide **2** and RGDL-lipopeptide **3**, reverse phase HPLC chromatograms for RGDK-lipopeptide **1**, RGEK-lipopeptide **2**, RGDK-lipopeptide **3** in two mobile phases, and the details of the HPLC conditions; ^{1}H NMR and mass spectral data for RGE-lipopeptide **2** and RGDK-lipopeptide **3**, methods for preparation of liposomes, details of transfection and FACS protocol, General methods and materials, results in the antibody saturation experiments and in vivo tumor regression experiments with RGDL-lipopeptide **3**, and details for antibody saturation experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Carmeliet, P. Angiogenesis in life, disease and medicine. *Nature* **2005**, *438*, 932–936.
(2) Hynes, R. O. Integrins: bidirectional, allosteric signaling machines. *Cell* **2002**, *110*, 673–687.
(3) Giancotti, F. G.; Ruoslahti, E. Integrin Signaling. *Science* **1999**, *285*, 1028–1032.
(4) van der, F. A.; Sonnenberg, A. Function and interactions of integrins. *Cell. Tissue Res.* **2001**, *305*, 285–298.
(5) Varner, J. A.; Emerson, D. A.; Juliano, R. L. Integrin alpha 5 beta 1 expression negatively regulates cell growth: reversal by attachment to fibronectin. *Mol. Biol. Cell* **1995**, *6*, 725–740.
(6) Friedlander, M.; Brooks, P. C.; Shaffer, R. W.; Kincaid, C. M.; Varner, J. A.; Cheresh, D. A. Definition of two angiogenic pathways by distinct alpha v integrins. *Science* **1995**, *270*, 1500–1502.
(7) Stupack, D. J.; Cheresh, D. A. Integrins and angiogenesis. *Curr. Top. Dev. Biol.* **2004**, *64*, 207–238.
(8) Brooks, P. C.; Montgomery, A. M.; Rosenfeld, M.; Reisfeld, R. A.; Hu, T.; Klier, G.; Cheresh, D. A. Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell* **1994**, *79*, 1157–1164.
(9) Brooks, P. C.; Strömblad, S.; Klemke, R.; Visscher, D.; Sarkar, F. H.; Cheresh, D. A. Antiintegrin alpha v beta 3 blocks human breast cancer growth and angiogenesis in human skin. *J. Clin. Invest.* **1995**, *96*, 1815–1822.
(10) Friedlander, M.; Theesfeld, C. L.; Sugita, M.; Fruttiger, M.; Thomas, M. A.; Chang, S.; Cheresh, D. A. Involvement of integrins alpha v beta 3 and alpha v beta 5 in ocular neovascular diseases. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 9764–9769.
(11) Bader, B. L.; Rayburn, H.; Crowley, D.; Hynes, R. O. Extensive Vasculogenesis, Angiogenesis, and Organogenesis Precede Lethality in Mice Lacking All αv Integrins. *Cell* **1998**, *95*, 507–519.
(12) Hodivala-Dilke, K. M.; McHugh, K. P.; Tsakiris, D. A.; Rayburn, H.; Crowley, D.; Ullman-Culleré, M.; Ross, F. P.; Coller, B. S.; Teitelbaum, S.; Hynes, R. O. Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. *J. Clin. Invest.* **1999**, *103*, 229–238.
(13) Reynolds, L. E.; Wyder, L.; Lively, J. C.; Taverna, D.; Robinson, S. D.; Huang, X.; Sheppard, D.; Hynes, R. O.; Hodivala-Dilke, K. M. Enhanced pathological angiogenesis in mice lacking beta3 integrin or beta3 and beta5 integrins. *Nat. Med.* **2002**, *8*, 27–34.
(14) Kim, S.; Bell, K.; Mousa, S. A.; Varner, J. A. Regulation of angiogenesis in vivo by ligation of integrin alpha5beta1 with the central cell-binding domain of fibronectin. *Am. J. Pathol.* **2000**, *156*, 1345–1362.
(15) George, E. L.; Georges-Labouesse, E. N.; Patel-King, R. S.; Rayburn, H.; Hynes, R. O. Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. *Development* 1993*119*, 1079–1091.
(16) Yang, J. T.; Rayburn, H.; Hynes, R. O. Embryonic mesodermal defects in alpha 5 integrin-deficient mice. *Development* **1993**, *119*, 1093–1105.
(17) Heckmann, D.; Meyer, A.; Marinelli, L.; Zahn, G.; Stragies, R.; Kessler, H. Probing integrin selectivity: rational design of highly active and selective ligands for the a5β1 and avβ3 intrgrin receptor. *Angew. Chem., Int. Ed.* **2007**, *46*, 3571–3574.
(18) Stragies, R.; Osterkamp, F.; Zischinsky, G.; Vossmeyer, D.; Kalkhof, H.; Reimer, U.; Zahn, G. Design and synthesis of a new class of selective integrin alpha5beta1 antagonists. *J. Med. Chem.* **2007**, *50*, 3786–3794.
(19) Meyer, A.; Auernheimer, J.; Modlinger, A.; Kessler, H. Targeting RGD recognizing integrins: drug development, biomaterial research, tumor imaging and targeting. *Curr. Pharm. Des.* **2006**, *12*, 2723–2747.
(20) Smallheer, J. M.; Weigelt, C. A.; Woerner, F. J.; Wells, J. S.; Daneker, W. F.; Mousa, S. A.; Wexler, R. R.; Jadhav, P. K. Synthesis and biological evaluation of nonpeptide integrin antagonists containing spirocyclic scaffolds. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 383–387.
(21) Zimmermann, D.; Guthöhrlein, E. W.; Malesević, M.; Sewald, K.; Wobbe, L.; Heggemann, C.; Sewald, N. Integrin $\alpha5\beta1$ ligands: biological evaluation and conformational analysis. *ChemBioChem.* **2005**, *6*, 272–276.
(22) Ruoslahti, E. RGD and other recognition sequences for integrins. *Annu. Rev. Cell Dev. Biol.* **1996**, *12*, 697–715.
(23) Triantafilou, K.; Takada, Y.; Triantafilou, M. Mechanisms of integrin-mediated virus attachment and internalization process. *Crit. Rev. Immunol.* **2001**, *21*, 311–322.
(24) Garrigues, H. J.; Rubinchikova, Y. E.; DiPersio, C. M.; Rose, T. M. Integrin alphaVbeta3 Binds to the RGD motif of glycoprotein B of Kaposi's sarcoma-associated herpesvirus and functions as an RGD-dependent entry receptor. *J. Virol.* **2008**, *82*, 1570–1580.
(25) Xiong, X.; Huang, Y.; Lu, W. L.; Zhang, X.; Zhang, H.; Nagai, T.; Zhang, Q. Intracellular delivery of doxorubicin with RGD-modified sterically stabilized liposomes for an improved antitumor efficacy: in vitro and in vivo. *J. Pharm. Sci.* **2005**, *94*, 1782–1793, and the references 16−20 cited therein.
(26) Koppitz, M.; Huenges, M.; Gratias, R.; Kessler, H. Synthesis of Unnatural Lipohilic *N*-(9*H*-Fluoren-9-ylmethoxy)carbonyl-Substituted Amino Acids and Their Incorporation into Cyclic RGD-Peptides: A Structure−Activity Study. *Helv. Chim. Acta* **1997**, *80*, 1280–1300.
(27) Hu, B.; Finsinger, D.; Peter, K.; Guttenberg, Z.; Bärmann, M.; Kessler, H.; Escherich, A.; Moroder, L.; Böhm, J.; Baumeister, W.; Sui, S. F.; Sackmann, E. Intervesicle cross-linking with integrin alpha IIb beta 3 and cyclic-RGD-lipopeptide. A model of cell-adhesion processes. *Biochemistry* **2000**, *39*, 12284–12294.
(28) Nasongkla, N.; Shuai, X.; Ai, H.; Weinberg, B. D.; Pink, J.; Boothman, D. A.; Gao, J. cRGD-functionalized polymer micelles for targeted doxorubicin delivery. *Angew. Chem., Int. Ed.* **2004**, *43*, 6323–6327.
(29) Hood, J. D.; Bednarski, M.; Frausto, R.; Guccione, S.; Reisfeld, R. A.; Xiang, R.; Cheresh, D. A. Tumor regression by targeted gene delivery to the neovasculature. *Science* **2002**, *296*, 2404–2407.
(30) Murphy, E. A.; Majeti, B. K.; Barnes, L. A.; Makale, M.; Weis, S. M.; Lutu-Fuga, K.; Wrasidlo, W.; Cheresh, D. A. Nanoparticle-mediated drug delivery to tumor vasculature suppresses metastasis. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 9343–9348.
(31) Karmali, P. P.; Valluripalli, V. K.; Chaudhuri, A. Design, syntheses and in vitro gene delivery efficacies of novel mono-, di-, and trilysinated cationic lipids: a structure−activity investigation. *J. Med. Chem.* **2004**, *47*, 2123–2132.
(32) Rajesh, M.; Sen, J.; Srujan, M.; Mukherjee, K.; Sreedhar, B.; Chaudhuri, A. Dramatic influence of the orientation of linker between hydrophilic and hydrophobic lipid moiety in liposomal gene delivery. *J. Am. Chem. Soc.* **2007**, *129*, 11408–11420.
(33) Cherny, R. C.; Honan, M. A.; Thiagarajan, P. Site-directed mutagenesis of the arginine-glycine-aspartic acid in vitronectin abolishes cell adhesion. *J. Biol. Chem.* **1993**, *268*, 9725–9729.
(34) Beauvais, A.; Erickson, C. A.; Goins, T.; Craig, S. E.; Humphries, M. J.; Thiery, J. P.; Dufour, S. Changes in the fibronectin-specific integrin expression pattern modify the migratory behavior of sarcoma S180 cells in vitro and in the embryonic environment. *J. Cell. Biol.* **1995**, *128*, 699–713.

JM800915Y