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molecular pharmaceutics

Single Subcutaneous Administration of RGDK-Lipopeptide:rhPDGF-B Gene Complex Heals Wounds in Streptozotocin-Induced Diabetic Rats

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Received November 13, 2008; Revised Manuscript Received March 11, 2009; Accepted March 31, 2009

Abstract: Development of effective therapeutics for chronic wounds remains a formidable clinical challenge. Deficiency of growth factors is of paramount importance among the multitude of factors contributing to the pathogenesis of diabetic wounds. Clinical interest has been witnessed in the past for exogenous applications of platelet derived growth factor B (PDGF-B) in chronic nonhealing wounds. However, accomplishing even modest favorable clinical effects in such topical applications requires large and repeated doses of PDGF-B proteins. Chronic wounds are being increasingly circumvented by gene therapy approach and to this end, cationic liposomes are emerging as promising nonviral carriers for delivering various growth factors encoding therapeutic genes to wound beds. However, as in case of topical application of growth factors, all the prior studies on the use of cationic liposomes in nonviral gene therapy of wounds involved repeated injections of cationic liposome:cDNA complexes over several weeks for ensuring complete wound healing. Herein, we show that a single subcutaneous administration of an electrostatic complex of rhPDGF-B plasmid, integrin receptor selective RGDK-lipopeptide **1** and cholesterol (as auxiliary lipid) is capable of healing wounds in streptozotocin-induced diabetic Sprague−Dawley rats (as model of chronic wounds). Western blot analysis revealed significant expression of rhPDGF-B in mouse fibroblast cells transfected with RGDK-lipopeptide **1**:rhPDGF-B lipoplex. The transfection efficiencies of the RGDK-lipopeptide **1** in mouse and human fibroblast cells preincubated with various monoclonal anti-integrin receptor antibodies support the notion that the cellular uptake of the RGDK-lipopeptide **1**:DNA complexes in fibroblast cells is likely to be selectively mediated by $\alpha 5\beta 1$ integrin receptors. Findings in the histopathological stainings using both hematoxylin and eosin (H & E) as well as Masson's Trichrome staining revealed a significantly higher degree of epithelization, keratization, fibrocollagenation and blood vessel formation in rats treated with RGDK-lipopeptide **1**:rhPDGF compared to those in rats treated with vehicle alone.

Keywords: RGDK-lipopeptide; PDGF-B growth factor; chronic wounds; liposomal gene delivery

Introduction

Development of effective therapeutics for chronic wounds remains a formidable clinical challenge despite the fact that chronic wounds account for 25−50% of total diabetic health costs annually representing billons of dollars worldwide.[1]

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10.1021/mp800231z CCC: $40.75
Published on Web 04/02/2009

Wound healing is a complex and highly integrated cascade of events involving four distinct but overlapping phases: hemostasis, inflammation, proliferation and remodeling.[2−4] Hemostasis begins immediately after the tissue is injured. As soon as the blood components spill into the site of injury, the blood platelets get exposed to collagen and other extracellular matrix components of the injured area. This contact triggers the platelets to release various clotting factors, platelet derived growth factors (PDGF) and transforming growth factor beta (TGF-β). Thereafter, in the inflammation phase, the body's inflammatory cells, e.g., neutrophils, migrate to the wound sites followed by migration of the various other immune cells to the wound site. During this inflammation stage, stimulated neutrophils release proteases and reactive oxygen species into the surrounding medium of the wound bed to combat assaults from the invading pathogenic microorganisms, and macrophages expedite the phagocytic removal of foreign materials and damaged tissues and release more PDGF and TGF-β. Once the wound bed is cleaned, fibroblasts migrate into the wound area and deposit new extracellular matrix thereby inducing formation of new granulation tissues and new blood vessels in the injured area in the proliferation stage. In this cellular proliferation stage, fibroblasts, endothelial cells and epithelial cells migrate to the wound site. The fibroblast cells produce the collagen needed for wound repair. The epithelial cells migrate from free edges of the wound tissue across the wound for re-epithelization followed by their proliferation at the periphery of the wound. In the final phage of wound remodeling, replacement of the granulation tissue with collagen fibers takes place followed by devascularization of the granulation tissue, which eventually leads to the formation of a scar over the wound area. The newly deposited extracellular matrix (e.g., collagen) becomes cross-linked and organized during this final remodeling stage.

All the above-mentioned cascade of orderly events are lost in cases of impaired or chronic wounds, such as diabetic wounds and pressure ulcers. Excessive infiltration by neutrophils with their associated reactive oxygen species (ROS) and degradative enzymes locks the chronic ulcers into a state of lasting inflammation. Among the multitude of factors contributing to the pathogenesis of chronic wounds, deficiency of growth factors is of paramount importance.[5,6] This is because wound proteases destroy the peptide growth factors at a fast rate.[7] Clinical interest has been witnessed in the past for exogenous applications of platelet derived growth factor B (PDGF-B) in chronic nonhealing wounds.[8−13] However, accomplishing even modest favorable clinical effects in such topical applications requires large and repeated doses of PDGF-B proteins.[8−11] Short shelf life and inefficient delivery to target cells are two additional concerns associated with direct topical administrations of growth factors. All these alarming impeding factors related to topical administrations of growth factors in treating chronic wounds are being increasingly circumvented by the gene therapy approach in which genes encoding various growth factors in complexation with efficacious gene carriers are administered for inducing expression of therapeutic protein, such as PDGF-B. To this end, cationic liposomes are emerging as promising nonviral carriers for delivering various growth factors encoding therapeutic genes to wound beds.[14−17] However, all the prior studies on the use of cationic liposomes in nonviral gene therapy of wounds involved repeated injections of cationic

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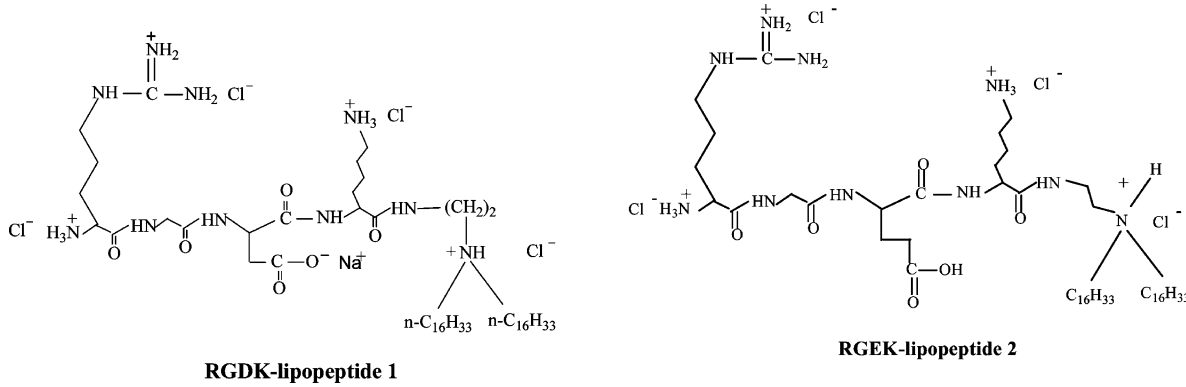

Figure 1. Structures of RGDK-lipopeptide **1** and RGEK-lipopeptide **2**.

liposome:cDNA complexes over several weeks for ensuring complete wound healing. Recently, we demonstrated that the RGDK-lipopeptide **1** (Figure 1) can selectively target genes to the proangiogenic $\alpha5\beta1$ integrin receptors.[18] Since fibroblasts, one of the important cells involved in the wound healing process, also express $\alpha5\beta1$ integrin receptors in their cell surface,[19,20] we envisioned that RGDK-lipopeptide **1** should be able to deliver growth factor encoded genes to the fibroblasts of the wound beds. Herein, we show that a single subcutaneous administration of an electrostatic complex of rhPDGF-B plasmid, the RGDK-lipopeptide **1** and cholesterol (as auxiliary lipid) is capable of healing wounds in streptozotocin-induced diabetic Sprague−Dawley rats (as model of chronic wounds) in 10 days.

Experimental Section

Animals and Cells. Sprague−Dawley (S/D) male rats (230−260 g) were obtained from National Institute of Nutrition, Hyderabad, India, and all the in vivo experiments were performed in accordance with the Institutional Biosafety and Ethical Committee guidelines using an approved animal protocol. 3T3 (mouse fibroblast) and WI38 (human lung fibroblast) cell lines were procured from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 50 μg/mL penicillin, 50 μg/mL streptomycin and 20 μg/mL kanamycin in a humidified atmosphere containing 5% CO_2.

Syntheses. RGDK-lipopeptide **1** and the control RGEK-lipopeptide **2** were synthesized as described recently[18] by covalent grafting of the RGD and RGE sequences, respectively, to the lysine residue of a monolysinated cationic amphiphile precursor readily available in our laboratory.

Plasmids and Reagents. pCMV-Luc and pCMV-SPORT-β-gal plasmids were generous gifts from Dr. Leaf Huang, University of North Carolina at Chapel Hill, NC, and Dr. Nalam Madhusudhana Rao, Centre for Cellular and Molecular Biology, Hyderabad, India, respectively. These plasmids were amplified in DH5α strain of *Escherichia coli*, isolated by alkaline lysis procedure and finally purified by PEG-8000 precipitation as described previously.[21] The purity of plasmid was checked by A_{260}/A_{280} ratio (around 1.9) and 1% agarose gel electrophoresis. Mouse antihuman $\alpha5\beta1$ monoclonal antibody, mouse antihuman $\alpha v\beta3$ monoclonal antibody, mouse antihuman $\alpha v\beta5$ monoclonal antibody and rabbit antimouse $\alpha5\beta1$ monoclonal antibody were purchased from Chemicon, USA.

Cloning of Recombinant Human PDGF. PDGF-B cDNA was synthesized from mRNA isolated from HUVEC-2 cells (B. D. Biosciences) and amplified by RT-PCR as mentioned previously.[22] The PDGF-B gene was amplified from cDNA by using gene specific primers which contains *Bam*H1 restriction site in forward primer and *Eco*R1 site in reverse primer.

forward primer:
5′-CGGGATCCACCATGGCAAGCCTGGGTTCCC-3′
reverse primer:
5′-GGCGAATTCTCAGGTCACAGGCCGTGC-3′

The amplified PDGF-B gene and pCDNA3.1 vector (Invitrogen) were digested with *Bam*HI and *Eco*RI (New England

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Biolabs). The gel-purified digested PDGF-B gene was cloned into pCDNA3.1 as an *Bam*HI and *Eco*RI fragment. Successful construction of the pCDNA3.1-PDGF-B clone was confirmed by restriction digestion and DNA sequencing.

Preparation of liposomes and Lipid−DNA Complexes. Cationic lipids and cholesterol (in 2:1 molar ratio) in chloroform were dried under a stream of N_2 gas and vacuum-desiccated for a minimum of 6 h to remove residual organic solvent. The dried lipid film was hydrated in sterile deionized water (for in vitro) or 5% w/v glucose water (for in vivo) at cationic lipid concentration of 1 mM and 5 mM respectively, for a minimum of 12 h. Liposomes were vortexed to remove any adhering lipid film and probe sonicated until a clear translucent solution formed. For preparation of lipid−DNA complexes (in vivo), pDNA (50 μg) and cationic liposomes (using appropriate volumes of stock liposomes for preparing lipoplexes with the lipid:DNA molar ratio of 9:1) were diluted to 150 μL each with 5% w/v glucose solution in sterile 1.5 mL microfuge tubes separately. The pDNA solution was added to the liposomes, mixed properly by pipetting up and down a few times and kept at room temperature for 15−30 min before use.

Transfection of Mouse Fibroblast Cells. Cells were seeded at a density of 10,000 cells per well in a 96-well plate 18−24 h before transfection. 0.30 μg of pDNA was complexed with varying amounts of lipids (to give ± ratios of 0.3:1, 1:1, 3:1 and 9:1) in plain DMEM (total volume made up to 100 μL) for 20−30 min. The complexes were then added to the cells. After 3 h of incubation, 100 μL of DMEM with 10% FBS was added to the cells and the reporter gene activity was estimated after 48 h. Cells were washed with PBS (100 μL) and lysed in 50 μL of lysis buffer [0.25 M Tris-HCl (pH 8.0, 0.5% NP40]. The β-galactosidase activity per well was estimated by adding 50 μL of 2X-substrate solution (1.33 mg/mL of ONPG, 0.2 M sodium phosphate, pH 7.3, 2 mM magnesium chloride) to the lysate in a 96-well plate. Absorbance of the product ortho-nitrophenol at 405 nm was converted to β-galactosidase units by using calibration curve constructed using pure commercial β-galactosidase enzyme. The transfection values reported are the average values from two replicate experiments performed in the same plate on the same day. Each transfection experiment was performed three times on three different days. The day-to-day variation in transfection efficiency was mostly within 2−3-fold and was dependent on the cell density and condition of the cells.

Monoclonal Antibody Saturation Experiments. 3T3 cells were seeded at a density of 10,000 cells per well in a 96-well plate 18−24 h before the transfection. Cells were preincubated with 50 μL of three different monoclonal antibodies (at a dilution of 1:25 in DMEM) for 45 min at room temperature. After 45 min, medium containing the antibody from the cells were taken out. Then a fresh amount of 50 μL of three different monoclonal antibodies (at a dilution of 1:25 in DMEM) were readded to the cell wells. 50 μL of lipopeptide:DNA complexes (containing 9:1 ± charge ratio) was added to each well. After 1 h incubation at 37 °C, medium was discarded and 200 μL of 10% complete medium was added to each well. The reporter gene expression was monitored after 48 h. Each transfection experiment was conducted in duplicate or in triplicate on the same day. In WI38 cells, the luciferase reporter gene activity was estimated after 24 h. Medium from the cells was removed carefully and lysed in 50 μL of reporter lysis buffer. Care was taken to ensure complete lysis. To 8 μL of cell lysate was added 20 μL of Promega luciferase assay buffer in a polystyrene plate, and the luciferase activity per well was estimated using a FLx800 microplate luminescence reader (Bio-Tek instruments, INC, U.K.). Protein concentration in each well was determined by the modified Lowry method,[23] and the luciferase activity was expressed as the relative light units (RLU) per mg of the protein. Each transfection experiment was repeated three times on two different days. The transfection values reported were average values from three replicate transfection plates assayed on the same day.

rhPDGF-B Gene Expression in Mouse Fibroblast Cells by Western Blot. Mouse fibroblast cell lines were cultured on a 25 cm^2 tissue culture flask until they reached a confluency of about 70%. 3 μg of pDNA was complexed with required volume of 1 mM liposome solution in sterile water to give ± charge ratios of 9:1 in plain DMEM (total volume made up to 1 mL) for 10−15 min. The complexes were then added to the cells. After 3 h of incubation, medium was changed to complete medium containing 10% FBS. After 48 h the cells were detached from the flask using a cell scrapper. Whole cell lysates were prepared by lysing the cells as mentioned elsewhere.[24] Total protein content in each sample was determined by BCA method.[23] Cell lysates and pure PDGF protein were loaded and separated on a 7.5% polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membrane (Hybond-C extra, Amersham Biosciences, NJ) using wet blotting. Membrane was blocked for 1.5 h with 3% BSA solution in PBS-T (phosphate buffer saline containing 0.05% Tween-20). Blot was then incubated with polyclonal antibody raised against PDGF-BB of human origin in rabbit (Virchow Biotech) at 1:500 dilution overnight at 4 °C. After washing three times with PBS-T, the membrane was incubated with goat anti rabbit secondary antibody conjugated to horseradish peroxidase (Bangalore Genei, India) at 1:1000 dilutions for 60 min. After washing three times with PBS-T protein bands were visualized using TMB-Blotting methods with TMB (Pierec Biotechnology Inc., Pittsburgh, PA) according to the manufacturer's protocol.

Diabetic Sprague−Dawley (S/D) Rats Wound Model. S/D rats were made diabetic by a single injection of streptozotocin (STZ, Sigma, St Louis, MO) in citrate buffer, pH 4.5 (45 mg/kg, ip) after overnight fasting. Daily blood glucose

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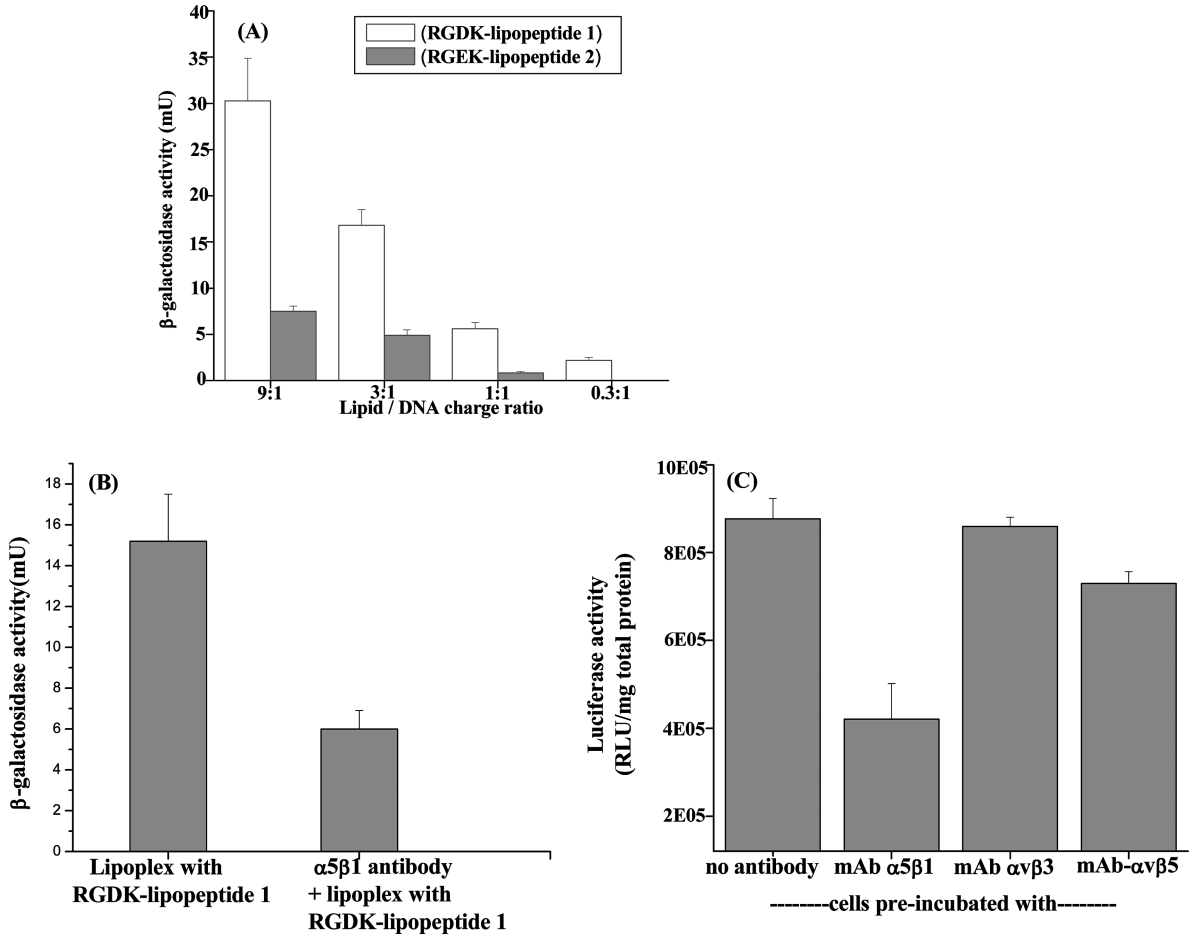

Figure 2. (A) In vitro transfection efficiencies of RGDK-lipopeptide **1** and RGEK-lipopeptide **2** in mouse fibroblast cells (3T3) using cholesterol as colipid (at lipid:cholesterol mole ratio of 2:1). (B) In vitro transfection efficiencies of RGDK-lipopeptide **1** in untreated mouse fibroblast cells (3T3) and in 3T3 cells preincubated with mouse α5β1 mAb. Units of β-galactosidase activity were plotted against the varying lipid to DNA (±) charge ratios. The *o*-nitrophenol formation (micromoles of *o*-nitrophenol produced per 5 min) was converted to β-galactosidase activity units using the standard curve obtained with pure commercial β-galactosidase. (C) In vitro transfection efficiencies of RGDK-lipopeptide **1** in untreated human lung fibroblast cells (WI38) and in WI38 cells preincubated with anti human integrin α5β1, αvβ3 and αvβ5 mAbs.

measurements were performed by blood analyzer (BAYER CORP., USA, Express plus, model no. 15065) for all animals using retro-orbital blood samples. Diabetic status was defined as blood glucose levels (nonfasting) higher than 300 mg/dL. Fourteen days after STZ treatment, the backs of the diabetic rats were shaved and the rats were anesthetized with ether solution. A 2.1 cm (radii) circular dorsal skin incision was produced with a scalpel down to the level of the loose subcutaneous tissues. For the first 48 h postwounding the animal was left undisturbed and the treatments were given as described in the figure legend of Figure 5. Measurement of wound areas was taken on every alternate day. The outline of the wound was traced as accurately as possible on a superimposed sheet of sterile cellophane. This outline was then transferred directly to a sheet of tracing paper and the area within it was determined by counting the number of the smallest squares on the tracing paper. The wound healing process was monitored by calculating the percent reduction in the wound area calculated with reference to wound area on day zero.

Histopathology. Skin samples of wound areas from all different groups were collected and fixed in 10% neutral buffer formalin. After overnight fixation, the samples were processed as per standard protocol to obtain 4 μm thick paraffin sections, which were then stained routinely with H & E. The sections were also stained with Masson's Trichrome for clear delineation of fibrocollagenous tissues, epidermises and muscles. The tissue sections were examined under Nikon E800 Eclipse microscope.

Statistical Analysis. Data are represented as mean ± SD and were compared among different groups using the Student *t* test. $p < 0.01$ was considered as significant.

Results

Integrin Receptor Specific Fibroblast Transfection by RGDK-Lipopeptide 1. Toward evaluating the potential of the RGDK-lipopeptide **1** in nonviral gene therapy of chronic wounds, first we measured its efficacy in transfecting Balb c

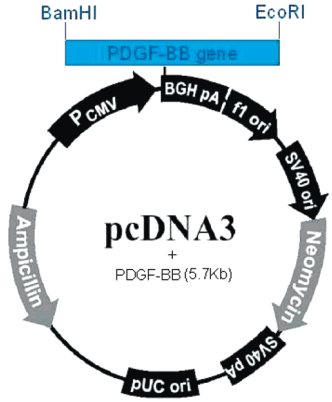

Figure 3. rhPDGF-B vector map.

mouse fibroblasts (3T3 cells, an important participating cell in wound healings) using p-CMV-SPORT-β-galactosidase plasmid DNA as the reporter gene. Consistent with our expectation, findings in the reporter gene expression assay revealed that the RGDK-lipopeptide **1** in combination with cholesterol as colipid (at 2:1 mol ratios of the RGDK-lipopeptide **1** and cholesterol) was efficacious in delivering genes to mouse fibroblast cells particularly at RGDK-lipopeptide **1**:DNA charge ratio of 9:1 (Figure 2, part A, white bars). Next, with a view to gain mechanistic insights into whether or not integrin receptors are indeed involved in the uptake of the RGDK-lipopeptide **1**:DNA complex by the mouse fibroblast cells, we repeated the in vitro transfection experiments in 3T3 cells using the control RGEK-lipopeptide **2** (Figure 1, part A) across the lipid:DNA charge ratios 9:1−0.3:1. RGD and not RGE is ligand for integrin receptors,[25] and thus, if integrin receptors are involved in the cellular uptake process in fibroblast cells, the transfection efficiency of the control RGEK-lipopeptide **2** is expected to be significantly lower than that of RGDK-lipopeptide **1**. Indeed this turned out to be the case. The transfection efficiencies of the control RGEK-lipopeptide **2** (Figure 2, part A, gray bars) were found to be significantly lower than those of the RGDK-lipopeptide **1** (white bars, Figure 2, part A) across the entire lipid:DNA charge ratios 9:1−0.3:1 thereby indicating the involvement of integrin receptors mediated cellular uptake of the RGDK-lipopeptide **1**:DNA complexes in mouse fibroblast cells. Recently, we have demonstrated that the RGDK-lipopeptide **1** can deliver genes into A549 (human lung carcinoma cells) selectively via $\alpha5\beta1$ integrin receptor.[18] 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. Thus, the cellular uptake of the lipopeptide:DNA complexes is unlikely to be mediated via either $\alpha v\beta3$ or $\alpha v\beta5$ 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.[18] Consistent with this prior finding, the transfection efficacies of RGDK-lipopeptide **1** in WI38 cells were significantly affected (by about 50%) only when cells preincubated with human monoclonal anti-$\alpha5\beta1$ integrin antibody (Figure 2, part C).

Figure 4. Representative Western blot for expression of PDGF-BB protein in mouse fibroblast (3T3) cells. Lane 1: Pure PDGF-BB protein. Lane 2: Lysate of the cells transfected with RGDK lipopeptide **1** and rhPDGF-B plasmid DNA. Lane 3: Lysate of the cells transfected with RGDK lipopeptide **1** and the control β-gal plasmid DNA. Lane 4: Lysate of the untransfected cells.

rhPDGF-B Expression in Mouse Fibroblast by RGDK-Lipopeptide 1. After demonstrating the $\alpha5\beta1$ integrin receptor specific gene transfer characteristics of the RGDK-lipopeptide **1**, we evaluated its efficiencies in delivering PDGF-growth factor encoded plasmid DNA (rhPDGF-B, the construction map shown in Figure 3) to mouse fibroblasts (3T3 cells). Western blot analysis of the transfected cell lysates revealed significant expression of rhPDGF-B in mouse fibroblast cells transfected with RGDK-lipopeptide **1**:rhPDGF-B complex (Figure 4). Importantly, neither the lysates of 3T3 cells transfected with electrostatic complex of RGDK-lipopeptide **1** and pCMV-β-Gal plasmid (the control plasmid DNA containing no encoded PDGF-B proteins) nor the lysates of the untransfected 3T3 cells showed any expression of rh-PDGF-B proteins (Figure 4, lanes 3 and 4, respectively). Thus, the findings in the Western blot experiments, taken together, convincingly demonstrated the efficiencies of the RGDK-lipopeptide **1** in delivering rhPDGF-plasmid to mouse fibroblasts cells.

Healing of Wounds in Streptozotocin-Induced Diabetic Rats. The significant expression of rhPDGF-BB in mouse fibroblast cells transfected with the RGDK-lipopeptide **1**:rhPDGF-B (Figure 4) prompted us to conduct preclinical studies for evaluating the systemic potential of the RGDK-lipopeptide **1** in healing wounds in diabetic rats (as a model for diabetic wounds). With a view to address the involvement of integrin receptor mediated cellular uptake of rhPDGF-B gene under systemic setup, we have carried out the wound healing experiments using lipoplexes of both RGDK-lipopeptide **1** (integrin ligand) and the control RGEK-lipopeptide **2** (not a ligand for integrin receptor). As summarized in Figure 5A, the rate of wound healing in S/D rats single time subcutaneously injected with RGDK-lipopeptide **1**:rhPDGF-B plasmid DNA complex in 5% glucose solution was much faster than that for rats singly injected with RGEK-lipopeptide **2**:rhPDGF-B plasmid DNA complex in 5% glucose. In order to probe the role of only liposomal vector prepared with RGDK-lipopeptide **1** in wound healing, if any, we also carried out the wound healing experiment with liposomes of RGDK-lipopeptide **1** without using rhPDGF-B plasmid DNA. As shown in Figure 5A, the rate of wound healing for liposomes of RGDK-lipopep-

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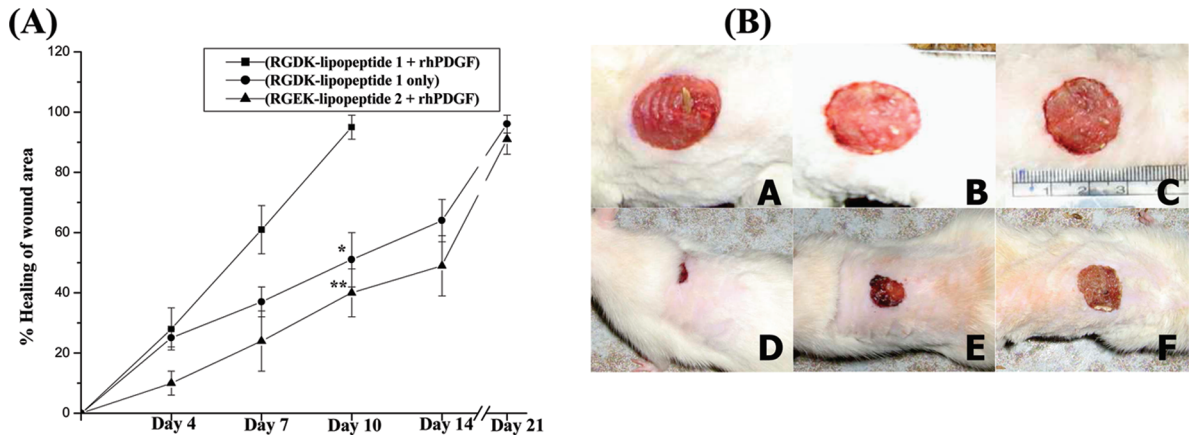

Figure 5. (A) The relative wound healing efficiency profiles in diabetic S/D rats injected with RGDK-lipopeptide **1**:rhPDGF-B complex (■, $n = 5$); RGDK-liposome only (●, $n = 4$, $^{*}P < 0.01$) and RGEK-lipopeptide **2**:rhPDGF-B complex (▲, $n = 4$, $^{**}P < 0.01$) in diabetic S/D rats. (B) Representative photographs of the wounds of diabetic S/D rats at the time of wounding and on 10th day after treatment. Wound sizes for diabetic S/D rats at the time of wounding (A−C); wound sizes on the 10th day after treatment for diabetic S/D rats treated with: RGDK-lipopeptide:rhPDGF-B complex (D); the control RGDK-liposome only (E) and the control RGEK-lipopeptide **2**:rhPDGF-B complex (F).

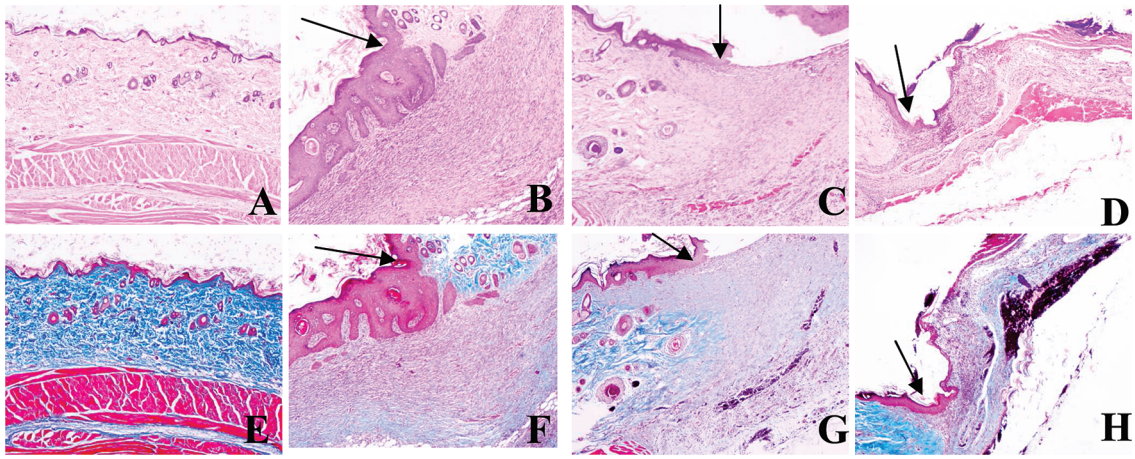

Figure 6. Subcutaneous administration of the RGDK-lipopeptide **1**:rhPDGF-B lipoplexes on the wound beds of streptozotocin-induced diabetic rats stimulates degree of re-epithelialization, fibrocollagen and keratin formation as well as blood vessel formation. Histopathological stainings for representative tissue sections: diabetic rats on the day of wounding (A and E); diabetic rats treated with RGDK-lipopeptide **1**:rhPDGF-B lipoplexes (B and F); diabetic rats treated with liposomes of RGDK-lipopeptide **1** (C and G); diabetic rats treated with RGEK-lipopeptide **2**:rhPDGF-B lipoplexes (D and H).

tide **1** only was much slower than that for the RGDK-lipopeptide **1**:rhPDGF-B lipoplex. The rates of wound healing were also found to be significantly slower when naked rhPDGF-B plasmid DNA as well as only buffer (5% aqueous glucose solution) were administered subcutaneously (data not shown). Representative photographs taken on day 10 (Figure 5B) revealed essentially complete healing of wounds in 10 days for rats treated with a single dose of the RGDK-lipopeptide **1**:rhPDGF-B plasmid DNA complex (complete wound healing was accomplished in exactly 12 days).

Histopathology. Findings in the histopathological stainings for representative wound tissue sections prepared on day 10 after wound generation using both hematoxylin and eosin (H & E) as well as Masson's Trichrome staining are summarized in Figure 6. Significantly higher degree of epithelization, keratization, fibrocollagenation and blood vessel formation were observed in rats treated with RGDK-lipopeptide **1**:rhPDGF-B (Figure 6, parts B and F) compared to those in rats treated with control RGDK-liposomes only (Figure 6, parts C and G) and in rats treated with control RGEK-lipopeptide **2**:rhPDGF-B lipoplex (Figure 6, parts D and H). Parts A and E of Figure 6 refer to the corresponding histopathological stainings for representative tissue sections of diabetic rats on the day of wounding.

Discussion

A multitude of factors contribute to the pathogenesis of chronic wounds. The connective tissue matrices in the chronic wound beds are destroyed by the degradative enzyme collagenase (matrix metalloproteinase-8) secreted by the

neutrophils.[26,27] In addition, the neutrophils release the proteolytic enzyme elastase capable of destroying various growth factors including PDGF-B and TGF-β.[28] Another important marker of chronic wounds is excessive accumulation of reactive oxygen species (ROS) in the wound bed that further damage the cells and healing tissues.[29] Among the various growth factor deficiencies in the chronic wound beds, the deficiency of platelet derived growth factor-B (PDGF-B) is of prime importance. Chronic nonhealing dermal ulcers are deficient in PDGF-B, and enhanced levels of PDGF-B have been found in the serum of patients capable of healing their ulcers.[5,6] Unfortunately, since high concentrations of wound proteases destroy growth factors at a fast rate,[7] large and repeated doses are required for ensuring even modest clinical benefits through topical applications of PDGF-B proteins.[8−11] To this end, in the emerging gene therapeutic modalities for combating impaired wounds, various growth factor encoded genes mostly in complexation with a viral vector (as gene carrier) are being administered instead of direct applications of growth factors on wound beds.[30−33] In general, the gene transfer efficacies of viral vectors are superior to those of their nonviral counterparts. However, multiple biosafety related concerns have been raised against use of viral transfection vectors in gene therapy. For instance, it has been demonstrated that systemic administration of adenoviral vectors is associated with substantial and dose-limiting liver toxicity[34] presumably due to high concentration of the Ad cellular receptor, coxsackie and adenovirus receptor, in the liver.[35,36] In addition, many prior reports have shown that viral vectors are capable of (a) generating potentially replication competent virus through various recombination events with the host genome, (b) inducing inflammatory and adverse immunogenic responses and (c) causing insertional mutagenesis through random integration into the host genome.[37−42]

All the above-mentioned alarming biosafety concerns associated with use of viral vectors are increasingly making cationic liposomes (least immunogenic) as the nonviral transfection vector of choice in gene therapy.[43] Because of their least immunogenic nature, robust manufacture, ability to deliver large pieces of DNA and ease of handling and preparation techniques, cationic liposomes are also emerging as efficient nonviral gene transfer vehicles for delivering genes to wound beds.[14−17] Sun et al. demonstrated that topical and subcutaneous administration of a-FGF cDNA with cationic liposomes on a daily basis in diabetic mice with incisional wounds enhanced wound breaking strength and improved wound healing quality when compared to controls.[17] Using commercially available cationic transfection lipid, namely, 1,2-dimyristyloxypropyl-3-dimethyl-hydroxy-ethyl ammonium bromide (DMRIE), Jeschke et al. demonstrated the therapeutic benefits of delivering IGI-I and KGF-cDNAs to acute wound beds.[14−16] However, in all these prior

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studies, repeated injections of cationic liposome:cDNA complexes over several weeks were necessary to ensure complete wound healing. To this end, in the present investigation, we have demonstrated that a single subcutaneous injection of cationic liposomes of an integrin receptor targeting RGDK-lipopeptide **1** and rhPDGF-B plasmid DNA is capable of healing incisional wounds in streptozocin induced diabetic mice (as model of chronic wounds) in 10 days.

Recently, we have demonstrated that the cationic RGDK-lipopeptide **1** containing a lysine functionality immediately after the RGD sequence in its polar headgroup region can selectively target genes to the proangiogenic $\alpha5\beta1$ integrin receptor and tumor vasculature.[18] Since fibroblast cells also express $\alpha5\beta1$ integrin receptor on its cell surface,[19,20] we evaluated transfection efficiency of the RGDK-lipopeptide **1**:pCMV-β-Gal lipoplex in mouse fibroblast cells preincubated with commercially available mouse monoclonal anti-$\alpha5\beta1$ integrin antibody. The significantly reduced transfection efficiency in 3T3 cells preincubated with mouse anti-$\alpha5\beta1$ integrin mAb (Figure 2, part B) is consistent with $\alpha5\beta1$ integrin receptor mediated uptake of the RGDK-lipopeptide **1** lipoplex in mouse fibroblast cells too. Unfortunately, due to nonavailability of mouse monoclonal anti-$\alpha v\beta3$ and anti-$\alpha v\beta5$ integrin antibodies, we could not address the integrin receptor selectivity of the RGDK-lipopeptide **1** in mouse fibroblast cells. However, availability of all three human monoclonal antibodies (mAbs) permitted us to probe the integrin receptor selectivity of the RGDK-lipopeptide **1** in human fibroblast cell (WI38). Since human fibroblast cells are hard to transfect, we used more sensitive luciferase assay in evaluating the transfection efficiencies of the RGDK-lipopeptide **1** in WI38 cells preincubated with mAbs of human anti-$\alpha5\beta1$, anti-$\alpha v\beta3$ and anti-$\alpha v\beta5$ integrin antibodies. The findings depicted in Figure 2, part C, are consistent with the supposition that the cellular uptake of the RGDK-lipopeptide **1**:pCMV-Luc lipoplex in human lung fibroblast cells is mediated selectively through the $\alpha5\beta1$ integrin receptor. Consistent with our expectation, cell lysate of the 3T3 cells transfected with RGDK-lipopeptide **1**:rh-CMV-PDGF complex revealed expression of PDGF-B in Western blot analysis (Figure 4). This finding prompted us to carry out in vivo experiments toward evaluating the systemic potential of the RGDK-lipopeptide **1** in nonviral gene therapy of diabetic wound. In insulin-dependent diabetes mellitus, the primary lesion is destruction of pancreatic β-cells.[44] Since streptozotocin-induced diabetic rats mimick this condition, they are useful models for investigating impaired wound healing in the diabetic state.[45,46] The systemic potential of the RGDK-lipopeptide **1** for delivering growth factor encoded genes in nonviral gene therapy of diabetic wounds was evaluated in streptozotocin-induced diabetic rats as models of chronic nonhealing wounds. Most importantly, only a single subcutaneous administration of the electrostatic complex of the RGDK-lipopeptide **1** liposomes and rh-CMV-PDGF plasmid DNA healed the incisional wounds in streptozotocin-induced diabetic Sprague−Dawley rats in ten days (Figure 5, parts A and B). Importantly, the rate of healing of wounds treated with RGDK-lipopeptide **1**:rh-CMV-PDGF complexes was much faster than those treated with the control RGEK-lipopeptide **2**:rh-PDGF-B lipoplex and only RGDK-lipopeptide **1** liposomes containing no rh-PDGF-B plasmid DNA (Figure 5, parts A and B). Similarly, wound healing rates were found to be much slower for wounds treated with naked rh-PDGF-B plasmid and 5% aqueous glucose solutions compared to the wound treated with RGDK-lipopeptide **1**:rh-CMV-PDGF lipoplex (data not shown). One might argue that the observed slower wound healings in these latter two control groups (i.e., wounds treated with naked rh-PDGF-B plasmid and wounds treated with 5% aqueous glucose solutions) result from infection in these control groups compared to the group treated with RGDK-lipopeptide **1**:rh-CMV-PDGF lipoplex that might not have been infected. If such is the case, then the group treated with only liposomes of RGDK-lipopeptide **1** (without rh-PDGF B plasmid) should have also shown fast wound healing, which was not observed (Figure 5). Thus, the observed faster wound closures for wounds treated with RGDK-lipopeptide **1**:rh-CMV-PDGF lipoplex are unlikely to originate from any pronounced infection inhibitory property of the RGDK-lipopeptide **1**. Stated differently, our findings are consistent with the supposition that both integrin receptor mediated uptake of the RGDK-lipopeptide **1**:rhPDGF-B lipoplex and activity of the expressed rh-PDGF-B protein contribute to the observed diabetic wound healing. Degree of re-epithelialization, fibrocollagen and keratin formation as well as blood vessel formation in sectioned wound tissue were found to be significantly enhanced in wounds treated with RGDK-lipopeptide **1**:rh-CMV-PDGF complexes compared to wounds treated with the control RGEK-lipopeptide **2**:rh-PDGF-B lipoplex and only RGDK-lipopeptide **1** liposomes (without rh-PDGF-B plasmid DNA) (Figure 6). Thus, the findings summarized in Figures 5 and 6, taken together, convincingly demonstrate that RGDK-lipopeptide **1** holds systemic potential in treating diabetic wounds. Previously, it has been demonstrated that delivery of multiple genes is more effective than transfer of a single gene.[47] Future systemic studies would throw further insights into whether the presently described RGDK-lipopeptide **1** mediated delivery of multiple genes encoding various other

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growth factors involved in wound healing would provide further therapeutic benefits.

Conclusions

We have demonstrated that a single subcutaneous administration of the electrostatic complex of RGDK-lipopeptide **1** and rhPDGF-B plasmid is capable of healing incisional wounds in streptozotocin-induced diabetic rats (as model for chronic wounds). Western blot analysis revealed significant expression of rhPDGF-B in mouse fibroblast cells transfected with RGDK-lipopeptide **1**:rhPDGF-B lipoplex. The transfection efficiencies of the RGDK-lipopeptide **1** in mouse and human fibroblast cells preincubated with various monoclonal anti-integrin receptor antibodies support the notion that the cellular uptake of the RGDK-lipopeptide **1**:DNA complexes in fibroblast cells are likely to be selectively mediated by $\alpha 5\beta 1$ integrin receptors. Findings in the histopathological stainings using both hematoxylin and eosin (H & E) as well as Masson's Trichrome staining revealed significantly higher degree of epithelization, keratization, fibrocollagenation and blood vessel formation in rats treated with RGDK-lipopeptide **1**:rhPDGF-B compared to those in rats treated with vehicle alone. Given that all the prior reported nonviral liposomal formulations required repeated doses for complete healing of wounds, the presently described integrin receptor specific RGDK-lipopeptide **1** opens a new door for combating often intractable chronic wounds.

Acknowledgment. This work was supported by the Department of Science and Technology, Government of India, New Delhi, and the Council of Scientific and Industrial Research, Government of India, New Delhi. We thank Nalam M. Rao for providing the p-CMV-SPORT-β-Gal plasmid. J.B. and G.M. thank the Council of Scientific and Industrial Research, Government of India, New Delhi, for providing doctoral research fellowships.

MP800231Z