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A Pilot Study Evaluating Combinatorial and Simultaneous Delivery of Polyethylenimine-Plasmid DNA Complexes Encoding for VEGF and PDGF for Bone Regeneration in Calvarial Bone Defects

Sheetal D'Mello^{a,#}, Satheesh Elangovan^{b,#,*}, Liu Hong^c, Ryan D. Ross^d, D. Rick Sumner^d, and Aliasger K. Salem^{a,b,*}

^aDivision of Pharmaceutics and Translational Therapeutics, College of Pharmacy, University of Iowa, Iowa City, IA, 52242

^bDepartment of Periodontics, College of Dentistry, University of Iowa, Iowa City, IA, 52242

^cDepartment of Prosthodontics, University of Iowa College of Dentistry, Iowa City, IA, USA

^dDepartment of Anatomy and Cell Biology, Rush Medical College, Chicago, IL, USA

Abstract

Gene therapy is a promising strategy to deliver growth factors of interest locally in a sustained fashion and has the potential to overcome barriers to using recombinant protein therapy such as sustainability and cost. Recent studies demonstrate the safety and efficacy of non-viral delivery of plasmid DNA (pDNA) encoding a single growth factor to enhance bone healing. This pilot study is aimed at testing a non-viral gene delivery system that can deliver two different plasmids encoding two different growth factors. Polyethylenimine (PEI), a cationic polymer, was utilized as a gene delivery vector and collagen scaffold was used as a carrier to deliver the PEI-pDNA complexes encoding platelet derived growth factor B (PDGF-B) and/or vascular endothelial growth factor (VEGF). Calvarial defects in rats were implanted with scaffolds containing PEI-pPDGF-B complexes, PEI-pVEGF complexes or containing both PEI-pPDGF-B and PEI-pVEGF complexes in a 1:1 ratio of plasmids. The results indicated that bone regeneration as measured using micro-CT and histological assessments was inferior in groups treated with PEI-(pPDGF-B + pVEGF) complexes, compared to defects treated with PEI-pPDGF-B complexes. This pilot study that explores the feasibility and efficacy of combinatorial non-viral gene delivery system for bone regeneration appears to provide a rationale for investigation of sequential delivery of growth factors at specific time points during the healing phases and this will be explored further in future studies.

*Corresponding Authors: Dr. Satheesh Elangovan, 801 Newton Road, S464, Department of Periodontics, University of Iowa College of Dentistry, Iowa City, Iowa -52242, satheesh-elangovan@uiowa.edu, Telephone: 319 335 7543, Fax: 319 335 7239, Dr. Aliasger K. Salem, 115 S. Grand Avenue, S228 PHAR, University of Iowa College of Pharmacy, Iowa City, IA 52242., aliasger-salem@uiowa.edu, Telephone: 319 335 8810, Fax: 319 335 9349.

#Joint first author

Keywords

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Both in orthopedics as well as in dentistry, there is an enormous need for developing novel biomaterials with improved bone regenerative capacity and predictability [1]. In dentistry, there are a multitude of conditions (periodontitis, ridge resorption, fracture or bone tumors) affecting the craniofacial complex in which bone loss is inevitable and predictable bone regeneration is required in order to restore both function and esthetics [2]. Bone replacement grafts currently available for predictable bone regeneration are either overly expensive or require harvesting from a distant donor site leading to significant morbidity [3]. Moreover, their predictability is restricted to select clinical indications. Over the last few decades, identification of key molecules involved in bone development and fracture healing has led to the introduction and rapid expansion of bone biomimetic materials in dentistry [4]. One such advancement is the introduction and current usage of growth factors or morphogens such as platelet derived growth factor-BB (PDGF-BB) and bone morphogenetic protein-2 (BMP-2) in clinical dentistry. Several studies including *in vitro*, *in vivo* and human clinical trials testing the efficacy of growth factors have clearly underscored their potential in regenerating lost bone or periodontium, leading to their approval for select clinical use [5].

Following tissue injury, platelets release PDGF at the site of the injury that act on specific cell surface receptors enhancing cell migration (chemotaxis) and proliferation (mitogenesis) [5]. The chemotactic ability of PDGF has been demonstrated on several cell types including osteoblasts [6]. Vascular endothelial growth factor (VEGF), a growth factor known for its role in angiogenesis, is also involved in both intra-membranous and endochondral ossification [7]. This property has led to the development of delivery systems that releases these factors for bone tissue engineering applications [8]. Combining PDGF with VEGF was shown to enhance the maturation of the blood vessels and to dramatically reverse experimentally induced ischemia in animals [9, 10]. Furthermore, there is growing evidence that delivery of PDGF along with VEGF has a synergistic effect on bone regeneration that is greater than either factor alone [11, 12].

Growth factors though promising are not without drawbacks. Recombinant proteins are expensive and the supraphysiological dosage in which they are used (to compensate for their shorter duration of activity in the *in vivo* milieu) raises serious safety concerns. Increasingly, side effects of delivering proteins such as BMP-2 in higher doses for both indicated and off-label use are being reported [13]. One approach to overcome the shortcomings of protein-based approaches is gene therapy [14]. Gene delivery allows targeted and controlled synthesis of gene products and proteins produced endogenously by this approach have been shown to be less altered and therefore less immunogenic [15]. Gene therapy studies conducted in animals using viral vectors delivered through a traditional *ex vivo* or an *in vivo* approach successfully demonstrated the feasibility and effectiveness of delivering *PDGF-B* genes in animal models [16–18]. In addition to periodontal regeneration, delivery of *PDGF-B* genes using viral vectors in animals has been shown to accelerate bone regeneration

around dental implants in a peri-implant bone loss model [19]. Even with its proven efficacy in animals, conducting human clinical trials and ultimately translating viral gene therapy into clinical settings, especially for non-lethal conditions can be extremely challenging [15]. In spite of lower transfection efficiency, non-viral vectors are safer and clinically translatable for bone regeneration applications. Once inside the target cells, the plasmid DNA (pDNA) containing non-viral vector is processed in the endosome and/or lysosome, and pDNA is released into the cytoplasm by a mechanism called endosomal escape [20]. The released pDNA translocates from the cytoplasm to the nucleus through the nuclear pores reaching the extra chromosomal space where it acts as local protein machinery [21].

We have recently reported that delivering pDNA encoding PDGF-B using non-viral vectors resulted in significant bone regeneration [22]. The new bone formation was 44x and 14x more, when the collagen scaffold carriers containing polyethylenimine (PEI)-pPDGF-B complexes were implanted into artificially created calvarial defects in rats, when compared to scaffold alone or empty defect groups, respectively. As a logical next step, in this study we explored the possibility of simultaneously delivering PDGF-B and VEGF plasmids from the same carrier system to evaluate the potential synergistic effects in bone regeneration. To our knowledge, this is the first description of delivery of multiple plasmids encoding different genes from a completely non-viral gene delivery system for bone regeneration.

Fabrication and *in vitro* characterization of PEI-pDNA complexes were performed as described previously [22]. Briefly, PEI (branched, 25 kDa, Sigma-Aldrich, St. Louis, MO)-pDNA (6.4 Kb pDNA encoding *luciferase* reporter gene, Vical, Inc., San Diego, CA) complexes were fabricated at amine (N) to phosphate (P) ratios of 1, 5, 10, 15, and 20, and characterized for size, surface charge, *in vitro* cytotoxicity and transfection efficacy in human bone marrow stromal cells (BMSCs; American Type Culture Collection, ATCC, Manassas, VA). The complexes prepared at an N/P ratio of 10 were 102 ± 2 nm in size with a positive surface charge of 37 ± 1 mV as measured using a Zetasizer Nano-ZS (Malvern Instruments, Westborough, MA). It was found that the transfection efficiency of pDNA complexes with PEI prepared at N/P ratio of 10 in BMSCs (1 μ g pDNA, seeding density of 80,000 cells/well in 24-well plates) was significantly greater than that measured for complexes prepared at all other N/P ratios as determined by luciferase expression using a standard luciferase assay system (Promega Corporation, Madison, WI) [22]. Complexes prepared at an N/P ratio of 10 also displayed low cytotoxicity in BMSCs (1 μ g pDNA, seeding density of 10,000 cells/well in 96-well plates) as assessed by the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) cell viability assay (Cell Titer 96 AQueous One Solution cell proliferation assay, Promega Corporation). In our study, only the PEI-pDNA complexes prepared at N/P ratio of 10 displayed efficient transfection of BMSCs with relatively lower cell toxicity when compared to complexes prepared at other N/P ratios. As a result, the PEI-pDNA complexes used in the *in vivo* experiments were prepared at N/P ratio of 10 [22]. The transgene nuclear delivery potential of the PEI-pPDGF-B (~ 4.9 Kb pDNA, Origene Technologies, Inc., Rockville, MD) and PEI-pVEGF (~ 4.9 Kb pDNA, Origene Technologies, Inc.) complexes prepared at N/P ratio of 10 and the subsequent expression of PDGF-BB and VEGF, respectively, was assessed in BMSCs (1 μ g pDNA each, seeding density of 80,000 cells/well in 24-well plates

incubated for 48 hours) using the PDGF-BB and VEGF ELISA kits (Quantikine, R & D Systems, Minneapolis, MN).

The surgical procedures were approved by and performed according to guidelines established by the University of Iowa Institutional Animal Care and Use Committee. To gain insights into the synergistic effects of delivering PDGF-B and VEGF plasmids, we utilized a calvarial defect model in rats and incorporated the following five groups: (a) empty defect, (b) empty collagen scaffold, (c) PEI-pPDGF-B complex-loaded scaffold, (d) PEI-pVEGF complex-loaded scaffold, and (e) PEI-(pPDGF-B + pVEGF) complex-loaded scaffold in a 1:1 ratio of plasmids. The *in vivo* bone regenerative capacity of the gene delivery system was investigated in two 5 mm diameter $\times$ 2 mm thickness critical-sized calvarial defects in each of the fourteen week-old male Fisher 344 rats (Charles River Laboratories International, Inc., Wilmington, MA) used in the study. Different animals were used for different treatment groups. For the PDGF group we had 6 animals (1 defect per animal) and for VEGF and PDGF+VEGF group, we had 3 animals each (1 defect per animal). Prior to implantation into the defects, the scaffolds were cut to fit the diameter of the defect. The skin wound was then sutured and post-operative care followed as per the protocol. The animals were euthanized after four weeks and the parietal bone fractions containing the regions of interest (5 mm diameter) were analyzed for bone volume (BV) per total volume (TV) using three-dimensional microfocus x-ray microcomputed tomography (micro-CT) imaging (cone-beam micro-CT40, Scanco Medical AG, Switzerland) as described earlier [22]. For histological observations, the bone specimens were stained with Harris hematoxylin and eosin (H & E staining) in order to evaluate bone regeneration after four weeks *in vivo* implantation. The light microscopic (bright-field) examination of the sections was performed with an Olympus Stereoscope SZX12 as described earlier [22].

As demonstrated in Figure 1, BMSCs transfected with PEI-pPDGF-B and PEI-pVEGF complexes resulted in significantly higher levels of PDGF-BB and VEGF in the cell culture medium supernatants compared to cells treated with naked, uncomplexed PDGF-B and VEGF plasmids. These results confirmed that PEI-pDNA complexes can efficiently transfect BMSCs with therapeutic genes for bone regeneration. VEGF was secreted into the supernatant at $\sim 3\times$ higher concentration when compared to PDGF-BB when the cells were treated under otherwise identical experimental conditions. Figure 2 is a schematic of the proposed mechanism of treatment for bone regeneration. The PEI-pDNA complexes are formed as a result of electrostatic interactions between the positively charged PEI and the negatively charged pDNA. The net positively charged complexes (prepared at N/P ratio of 10, with sizes approximately 100 nm as observed by transmission electron microscopy (TEM) images) are then injected into the porous collagen scaffolds and lyophilized. The complexes may then release from the degrading matrix, transfecting the surrounding cells (Figure 2). In addition, cells may migrate into the porous matrix containing the immobilized complexes followed by their transfection by the complexes. Ultimately, these transfected cells produce PDGF-BB and VEGF which stimulates further cell migration (via chemotaxis), proliferation, differentiation, angiogenesis, tissue remodeling and mature bone formation [23, 24].

We next evaluated the capacity of different treatments to initiate and form mature bone tissue. After four weeks of *in vivo* implantation of the scaffolds, the regions of interest were examined using micro-CT scans and bone tissue volume fraction of the total tissue volume of interest (BV/TV) was quantified. As depicted in Figure 3, treatment with the collagen scaffold matrix containing PEI-pPDGF-B complexes showed complete coverage of the defect by the newly-formed bone tissue. The combined therapy with pPDGF-B and pVEGF and defects implanted with PEI-pVEGF complexes alone exhibited only partial bone regeneration along the edges of the created defects. Patchy, irregularly-shaped, minimal mineralized regions were observed within the defects. A significant difference was found in the distribution of BV/TV between the PEI-pPDGF-B complex-embedded scaffolds and the remaining treatments.

The amount of bone tissue regenerated and the distribution of mineralization observed with this analysis was further validated using histological analysis of standard H & E stained sections of the defect regions four weeks post-implantation (Figure 4). Histology data showed that while the gap between the healthy native bone edges was unfilled within the empty defects, the other groups with the exception of PEI-pPDGF-B complex-loaded scaffolds displayed bridging of the defect by both soft and hard tissue formation as indicated by the presence of collagen fibrils, blood vessels, cells (most likely fibroblasts, osteogenic and/or inflammatory), extracellular matrix, and some new bone at the edges. For the PEI-pPDGF-B complex-loaded scaffold treatments, complete bridging of the defect by new bone (arrows) was observed. We hypothesize that the decreased bone formation seen in the defects treated with PEI-(pPDGF-B + pVEGF) complex-loaded scaffolds might be due to several reasons. Combining the two different pDNA encoding PDGF-B and VEGF respectively was used at concentrations that ensured we were using a dose of pPDGF that we have previously demonstrated to significantly enhance bone regeneration. This eliminated the possibility that any reduction observed in the bone regeneration was due to a lowered dose of pPDGF and it doubled the PEI concentration which may have increased the cytotoxicity *in vivo* via the apoptotic cell death pathway [25]. Our more comprehensive current and future studies will need to evaluate the impact of dose and differing ratios of pPDGF and pVEGF to determine if and to what extent these two parameters change the overall levels of bone regeneration when the two plasmids encoding for VEGF and PDGF are delivered simultaneously.

It's also been shown that some isoforms of VEGF are known to competitively block PDGF dependent binding and activation of PDGF receptors [26, 27]. Activation of PDGF receptors by VEGF *in vitro* led to PDGF-like effects [26]. As a result of this cross-talk, the true effect of VEGF might not have occurred in our system, again underscoring the importance of a spatio-temporal release system. Moreover, during the bone fracture healing, PDGF is secreted first by the platelets as well the inflammatory cells followed by the release of VEGF [11, 28]. Therefore, in conjunction with our findings, it appears to indicate that sequential delivery of these growth factors will be necessary for optimal bone regeneration.

Bone tissue engineering and development is a dynamic, highly regulated and organized process that is typically driven by the action of multiple growth factors for mediation of different cellular activities such as cellular recruitment, mitosis and differentiation of MSCs

into osteoblasts [29–31]. In the sequential cascade of events involved in bone regeneration, transient PDGF action is critical for chemotaxis, growth, and differentiation of MSCs in the early healing phase [32]. This can later be followed by prolonged, sustained angiogenic action of VEGF throughout the bone tissue healing process. Because PDGF-BB and VEGF each have distinct actions in bone formation, accordingly controlling their release may promote and induce more bone regeneration. This can be accomplished by delivering PDGF-BB and VEGF from a single novel composite collagen scaffold capable of differential release kinetics. This proposed strategy that would need to leverage and incorporate the advantages of controlled drug delivery systems [33–39], might be able to abolish or reduce the aforementioned concerns regarding simultaneous dual delivery of PDGF-BB and VEGF.

In this study, we showed that PEI was able to successfully transfect *PDGF-B* and *VEGF* genes into BMSCs. However, the bone regeneration efficacy was superior when the calvarial defects were treated with PEI-pPDGF-B complexes alone when compared with defects treated with PEI-(pPDGF-B + pVEGF) complexes. This study provides new insights into the possibility of developing combinatorial non-viral gene delivery system for bone regeneration and highlights the probable importance of temporal delivery of specific growth factors at specific time points in the healing process. We are currently exploring the sequential delivery of plasmids encoding two or more growth factors to promote bone regeneration and the use of other agents such as copper ions to promote vascularization [40].

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Figure 1. Detection of PDGF-BB and VEGF proteins in cell supernatants by ELISA after BMSC transfection with PEI-pPDGF-B and PEI-pVEGF complexes, respectively ($n = 3$, $***p < 0.001$). The data were compared by ANOVA, followed by a Tukey post-test analysis (Prism 5.0, GraphPad Software Inc., San Diego, CA). The differences between the groups were considered to be statistically significant at $p < 0.05$.

Figure 2. Schematic of the gene delivery system along with TEM of the complexes prepared at N/P ratio of 10 along with the proposed mechanism of action for bone defect repair.

Figure 3.

Representative micro-CT images of bone specimens demonstrating the extent of regenerated bone in the calvarial defects at four weeks in (a) untreated, open defects; (b) defects filled with empty collagen scaffolds; (c) defects filled with PEI-pPDGF-B complex-loaded scaffolds; (d) defects filled with PEI-pVEGF complex-loaded scaffolds; (e) defects filled with PEI-(pPDGF-B + pVEGF) complex-loaded scaffolds; and (f) regenerated bone volume fraction. Each defect within an animal was considered as being independent ($n = 3$, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$). The data were compared by ANOVA, followed by a Tukey post-test analysis (Prism 5.0, GraphPad Software Inc., San Diego, CA). The differences between the groups were considered to be statistically significant at $p < 0.05$.

Figure 4.

Representative histology sections of bone specimens showing the extent of regenerated bone in the calvarial defects at four weeks in (a) untreated, open defects; (b) defects filled with empty collagen scaffolds; (c) defects filled with PEI-pPDGF-B complex-loaded scaffolds; (d) defects filled with PEI-pVEGF complex-loaded scaffolds; (e) defects filled with PEI-(pPDGF-B + pVEGF) complex-loaded scaffolds (n = 3). OB = old bone and NB = new bone. Note the complete bridging of the bone defects by the regenerated tissue in the group treated with the PEI-pPDGF-B complex-loaded scaffolds as indicated by the arrows. Scale bar, 50 µm.