Journal of Controlled Release 248 (2017) 53–59

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Regeneration of bone using nanoplex delivery of FGF-2 and BMP-2 genes in diaphyseal long bone radial defects in a diabetic rabbit model

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ARTICLE INFO

Article history:
Received 16 August 2016
Received in revised form 7 October 2016
Accepted 4 January 2017
Available online 7 January 2017

Keywords:
Bone morphogenetic protein-2 (BMP-2)
Fibroblast growth factor-2 (FGF-2)
Diabetes mellitus
Polyethylenimine
Non-viral gene delivery
Bone regeneration

ABSTRACT

Bone fracture healing impairment related to systemic diseases such as diabetes can be addressed by growth factor augmentation. We previously reported that growth factors such as fibroblast growth factor-2 (FGF-2) and bone morphogenetic protein-2 (BMP-2) work synergistically to encourage osteogenesis *in vitro*. In this report, we investigated if BMP-2 and FGF-2 together can synergistically promote bone repair in a leporine model of diabetes mellitus, a condition that is known to be detrimental to union. We utilized two kinds of plasmid DNA encoding either BMP-2 or FGF-2 formulated into polyethylenimine (PEI) complexes. The fabricated nanoplexes were assessed for their size, charge, *in vitro* cytotoxicity, and capacity to transfect human bone marrow stromal cells (BMSCs). Using diaphyseal long bone radial defects in a diabetic rabbit model it was demonstrated that co-delivery of PEI-(*pBMP-2* + *pFGF-2*) embedded in collagen scaffolds resulted in a significant improvement in bone regeneration compared to PEI-*pBMP-2* embedded in collagen scaffolds alone. This study demonstrated that scaffolds loaded with PEI-(*pBMP-2* + *pFGF-2*) could be an effective way of promoting bone regeneration in patients with diabetes.

1. Introduction

Certain systemic diseases capable of impairing glucose metabolism can have profound deleterious effects on bone metabolism and bone healing [1]. Chronic diabetes mellitus (DM) is associated with decreased bone mineral density [2] and increased risk of fractures [3]. Individuals with DM have a significantly increased fracture risk compared with non-diabetic individuals [4]. Furthermore, they have an increased incidence of non-union after fracture, delayed union, and pseudarthrosis [5,6]. Several animal studies have documented that the animals with spontaneous DM or chemically induced DM have impaired callus formation as illustrated by reduced material strength properties of the callus compared with matched controls [7–10]. The cause of the altered biology at a diabetic fracture site remains unknown but is likely to be multifactorial with factors such as protein deficiency, neuropathy, small vessel deficiency [11] and increased rates of cartilage resorption all being potential contributors [12]. The necessity for better therapeutics stimulating bone regeneration and the healing of fractures in patients with chronic systemic conditions such as DM has resulted in the introduction of novel biomaterials, biomimetic factors, custom-fit printed scaffolds and cell based approaches in medicine [13]. One such development is the current clinical use of locally applied recombinant proteins that include bone morphogenetic protein-2 (BMP-2) or fibroblast growth factor-2 (FGF-2) for promoting osteogenesis. There have been a few studies that have explored the effect of locally applied recombinant human BMP-2 and FGF-2 to achieve bone regeneration in DM [14,15] and non-DM bone defect models [16–18]. It has been shown that BMP-2 treatment of fractures in a diabetic setting increased callus formation, vascularity and mechanical properties [14]. It has also been reported that intermittent FGF-2 treatment increased expression of transforming growth factor-beta (TGF-β) and increased callus formation at a fibula fracture site in diabetic rats [15].

BMP-2 is member of the TGF-β superfamily, which mediates multiple biological processes including regulation of bone formation [19]. BMP-2 is produced by osteoblastic cells and transduces its signals into the nucleus by binding to type I receptors (BMPR I) that then recruit type II receptors (BMPR II). BMP receptors then propagate their signals by phosphorylating several SMAD proteins (SMAD1, 5 or 8), which leads to upregulation of runt-related transcription factor 2 (Runx2) [20]. In addition, FGF-2 is also highly expressed in osteoblasts which modulates the proliferation and differentiation of mesenchymal stem cells [21]. FGF-2 signals through FGF receptor tyrosine kinases (FGFR1 to FGFR4). In osteoblasts, the interactions between FGF-2 and its receptors induces autophosphorylation of the receptors, which in turn activates and recruits FRS2 [22], src, ras, raf and extracellular signal-

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http://dx.doi.org/10.1016/j.jconrel.2017.01.008
0168-3659/

related kinase (ERK) [23], which leads to an increase in *Runx2* transcription [24]. Runx2 is a critical transcription factor in osteoblast precursor proliferation and differentiation [25]. Activation of Runx2 leads to increased expression of osteoblast-specific proteins, such as alkaline phosphatase and osteocalcin at different stages of osteogenesis, both of which promote bone formation [26]. *In vivo*, multiple growth factors act synergistically toward their respective target cells. For example, there is cooperation between BMP-2 and FGF-2 which act synergistically to enhance osteogenesis by promoting Runx2 transcription, through the activation of SMAD and ERK signaling pathways [27]. Our previous study revealed the synergistic effect on osteogenesis of the combinatorial treatment of *BMP-2* and *FGF-2 in vitro* [28]. Recombinant human protein growth factor based therapies have observable therapeutic effects. However, recombinant human proteins are expensive to manufacture and due to their short half-lives, there is a need for supraphysiological dosages for them to be clinically effective [29]. It has been shown that such high doses of recombinant human protein is associated with several side effects such as soft tissue swelling and ectopic bone formation [30]. Non-viral gene therapy is a promising alternative to protein based therapies [31]. It was shown that non-viral delivery of *BMP-2* and *FGF-2* genes enhanced upregulation of *Runx2* and *osteocalcin* transcription, which led to significantly enhanced mineralization in transfected cells *versus* non-transfected controls [28].

To our knowledge, no published study exists examining the synergistic effect of non-viral gene delivery of plasmids independently encoding BMP-2 and FGF-2 on the promotion of fracture healing in a chronic diabetic animal model. We hypothesized that non-viral gene delivery of *BMP-2* and *FGF-2* will synergistically attenuate the deleterious effects of DM on healing at the bone defect site. In the present study, well characterized nanoplexes were prepared by utilizing a cationic polymer, polyethylenimine (PEI), to condense plasmid DNA encoding BMP-2 and FGF-2 through electrostatic interaction. It has been previously shown that these nanoplexes possess efficient transfection capability both *in vitro* [28] and *in vivo* [32]. Collagen scaffolds harboring PEI-(*pBMP-2* + *pFGF-2*) nanoplexes, improved bone regeneration rates and increased callus formation when implanted into rabbit radius metaphyseal defect sites compared to control groups.

2. Materials and methods

2.1. Materials

Alloxan monohydrate, branched PEI (mol. wt. 25 kDa) and the GenElute™ HP endotoxin-free plasmid maxiprep kit were purchased from Sigma-Aldrich® (St. Louis, MO). The BMP-2 ELISA kit was purchased from Quantikine® (R & D Systems®, Minneapolis, MN). Plasmid DNA (6.9 Kb) encoding BMP-2 protein (Catalog number: SC119392) and plasmid DNA (4.9 Kb) encoding basic fibroblast growth factor-2 protein (FGF-2) (Catalog number: SC118884) were purchased from Origene Technologies, Inc. (Rockville, MD). Human bone marrow stromal cells (BMSCs) were purchased from the American Type Culture Collection (ATCC®, Manassas, VA). Dulbecco's Modified Eagle's Medium (DMEM), trypsin-EDTA (0.25%, 1× solution) and Dulbecco's phosphate buffered saline (PBS) were purchased from Gibco® (Invitrogen™, Grand Island, NY). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals® (Lawrenceville, GA). Gentamycin sulfate (50 mg/mL) was purchased from Mediatech Inc. (Manassas, VA). Absorbable type-I bovine collagen was provided from Zimmer Dental Inc. (Carlsbad, *CA*). MTS cell proliferation assay reagent (Cell Titer 96 AQueous One Solution Cell Proliferation Assay) was purchased from Promega (Madison, WI).

2.2. Isolation of plasmid DNA (pDNA) encoding BMP-2, and FGF-2 and fabrication and characterization of PEI-pDNA nanoplexes

Competent *E. coli* DH5α™ were transformed with the relevant *pDNA* which was subsequently amplified, purified and analyzed for purity as previously described [33]. Nanoplexes were created at a molar ratio of PEI amine (N) to *pDNA* phosphate (P) groups of 10, to achieve optimal transfection efficacies [33]. PEI-*pDNA* nanoplexes were formed by mixing 500 μL PEI solution with 500 μL *pDNA* (*pBMP-2 or pFGF-2*) solution containing 50 μg *pDNA* and vortexing for 30 s. A 1 mL solution of *pFGF-2* and *pBMP-2* plasmids containing 50 μg of each plasmid was mixed well by vortexing, and 500 μL was added to a separate vial. Then 500 μL of an indicated PEI concentration was added to the 500 μL of plasmid solution and vortexed for 30 s. The final mixture, containing 25 μg of *pFGF-2* and 25 μg of *pBMP-2*, was incubated for 30 min at ambient temperature to permit the formation of complexes through electrostatic interaction between PEI (amine groups) and *pDNA* (phosphate groups). A volume of 20 μL (containing 1 μg *pDNA*) of theses complexes was subsequently used for biocompatibility and *in vitro* transfection experiments. Nanoplexes in water were characterized for their size and zeta-potential using a Zetasizer Nano-ZS (Malvern Instruments, Westborough, MA) as previously described [34].

2.3. Collagen scaffold characterization

Collagen scaffold surface morphology was studied using scanning electron microscopy (SEM, Hitachi Model S-4800, Japan). Briefly, the scaffolds were mounted on a SEM aluminum stub using double stick carbon tape. This was sputter-coated with gold-palladium using an argon beam K550 sputter coater (Emitech Ltd., Kent, England). Images were captured using the Hitachi S-4800 SEM operated at 3 kV accelerating voltage and a current of 10 lA.

2.4. Fabrication of nanoplex-embedded collagen scaffolds

After preparation of nanoplexes at N/P ratio of 10, the nanoplexes were injected, using a sterile 28 gage needle, into the collagen scaffolds (hand cut into 5 mm × 5 mm). Afterwards, the nanoplex-embedded collagen scaffolds were lyophilized.

2.5. Cell culture

BMSCs were maintained in DMEM supplemented with 10% FBS and 1% gentamycin (50 μg/mL) in a humidified incubator (Sanyo Scientific Autoflow, IR direct heat CO_2 incubator) at 37 °C containing 95% air and 5% CO_2. Cells were cultured on 75 cm^2 polystyrene cell culture flasks (Corning, NY, USA) and sub-cultured (sub-cultivation ratio of 1:9) after 80–90% confluence was achieved. BDMCs were used in experiments at passage numbers 4–6.

2.6. Assessment of cytotoxicity and transfection efficiency of PEI-pDNA (pBMP-2 and pFGF-2) nanoplexes in vitro using BMSCs

Cytotoxicity of *PEI-pDNA* nanoplexes, at the N/P ratio of 10, cultured with BMSCs was assessed with the Cell Titer 96 AQueous One Solution

Table 1
Size and Zeta Potential of nanoplexes prepared at N/P Ratio 10.

Nanoplexes	*Z*-Ave (d. nm.) ± SEM	PDI ± SEM	Zeta Potential (mV) ± SEM
PEI-*pBMP-2*	83.4 ± 0.8	0.03 ± 0.01	34.9 ± 0.3
PEI-*pFGF-2*	90.5 ± 1.4	0.13 ± 0.02	35.6 ± 0.2
PEI-(*pBMP-2* + *pFGF-2*)	116.4 ± 1.1	0.08 ± 0.03	30.3 ± 0.3