Platelet-derived growth factor-B gene delivery sustains gingival fibroblast signal transduction

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Background and Objective: Platelet-derived growth factor-BB is a potent mediator of tooth-supporting periodontal tissue repair and regeneration. A limitation of the effects of topical platelet-derived growth factor-BB application is its short half-life *in vivo.* Gene therapy has shown strong promise for the long-term delivery of platelet-derived growth factor in both skin ulcer healing and periodontal tissue engineering. However, little is known regarding the extended effects of platelet-derived growth factor-B on cell signaling via gene delivery, especially at the level of phosphorylation of intracellular kinases. This study sought to evaluate the effect of gene transfer by Ad-PDGF-B on human gingival fibroblasts (HGFs) and the subsequent regulation of genes and cell-surface proteins associated with cellular signaling.

Material and Methods: HGFs from human subjects were treated by adenoviral PDGF-B, PDGF-1308 (a dominant negative mutant of *PDGF*) and recombinant human platelet-derived growth factor-BB, and then incubated in serum-free conditions for various time points and harvested at 1, 6, 12, 24, 48, 72 and 96 h. Exogenous *PDGF-B* was measured by RT-PCR and Western blot. Cell proliferation was evaluated by [methyl-³H]thymidine incorporation assay. We used proteomic arrays to explore phosphorylation patterns of 23 different intracellular kinases after *PDGF-B* gene transfer. The expression of α and β PDGFR and Akt were measured by Western blot analysis.

Results: Sustained *in vitro* expression of *PDGF-B* in HGFs by Ad-PDGF-B transduction was seen at both the mRNA and protein levels. Compared to rhPDGF-BB and Ad-PDGF-1308, Ad-PDGF-B maintained cell growth in serum-free conditions, with robust increases in DNA synthesis. Gene delivery of *PDGF-B* also prolonged downregulation of the growth arrest specific gene (*gas*) *PDGFaR*. Of the 23 intracellular kinases that we tested in proteomic arrays, Akt revealed the most notable long-term cell signaling effect as a result of the over-expression of Ad-PDGF-B, compared with pulse recombinant human platelet-derived growth factor BB. Prolonged Akt phosphorylation was induced by treatment with Ad-PDGF-B, for at least up to 96 h.

Conclusion: These findings further demonstrate that gene delivery of *PDGF-B* displays sustained signal transduction effects in human gingival fibroblasts that are higher than those conveyed by treatment with recombinant human platelet-derived growth factor-**BB** protein. These data on platelet-derived growth factor gene delivery contribute to an improved understanding of these pathways that are likely to play a role in the control of clinical outcomes of periodontal regenerative therapy.

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Z. Lin¹, J. V. Sugai¹, Q. Jin¹, L. A. Chandler², W. V. Giannobile^{1,3,4}

¹Department of Periodontics and Oral Medicine and Center for Craniofacial Regeneration, School of Dentistry, University of Michigan, Ann Arbor, MI, USA, ²Tissue Repair Company, San Diego, CA, USA, ³Department of Biomedical Engineering, College of Engineering, University of Michigan, Ann Arbor, MI, USA and ⁴Michigan Center for Oral Health Research, Ann Arbor, MI, USA

William V. Giannobile, DDS, DMedSc, Department of Periodontics and Oral Medicine, University of Michigan, 1011 N. University Avenue – Room 3397, Ann Arbor, MI 48109-1078, USA Tel: (734) 764 1562 Fax: (734) 763 5503 e-mail: wgiannob@umich.edu

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Platelet-derived growth factor, a member of a multifunctional polypeptide family, is composed of A, B, C and D polypeptide chains that form homodimeric or heterodimeric molecules (1). Two receptor isoforms with tyrosine kinase activity have been identified: platelet-derived growth factor- α receptor and platelet-derived growth factor- β receptor (1). Plateletderived growth factor binding to receptors activates a variety of intracellular signal molecules, such as phosphatidylinositol-3-kinase and Akt/ PKB, and subsequently exerts its biological effects on cell migration, proliferation, extracellular matrix synthesis and anti-apoptosis (2-7).

Platelet-derived growth factor-BB, which binds both α and β plateletderived growth factor receptors, promotes wound healing in both hard and soft tissues, such as skin and periodontium. Platelet-derived growth factor- α and - β receptors are induced in human gingival fibroblasts and toothsupporting periodontal ligament cells during periodontal tissue repair (8,9). In addition, platelet-derived growth factor initiates tooth-supporting periodontal ligament cell chemotaxis (10), mitogenesis (11), matrix synthesis (12,13) and attachment to tooth dentinal surfaces (14). In an experimental model of periodontitis in macaques, platelet-derived growth factor-BB was shown to promote new attachment and new bone formation in vivo (15). Furthermore, it has been recently shown that the application of platelet-derived growth factor-BB in an osteoconductive scaffold promotes periodontal regeneration in humans (16).

A limitation of the effects of topical platelet-derived growth factor application is its short half-life in vivo (17-19). Gene delivery is a candidate approach to overcome this problem. Delivery of platelet-derived growth factor by gene transfer has been shown to stimulate the mitogenesis and proliferation of gingival fibroblasts, periodontal ligament and tooth-lining cells (cementoblasts) more strongly than the continuous administration of platelet-derived growth factor protein in vitro (20,21). In terms of gene expression duration, in vitro studies have shown that adenovirus-encoded platelet-derived growth factor-A delivered to cells derived from periodontal tissues (gingival and periodontal ligament fibroblasts, cementoblasts and osteoblasts) elicits elevated and prolonged expression of the PDGF-A gene for at least 1 wk (20,22). Furthermore, transfer of the PDGF-A gene using adenovirus resulted in sustained tyrosine kinase phosphorylation and downregulation of the plateletderived growth factor-a receptor for at least 96 h in dermal fibroblasts (22). Our previous work has also shown that in vivo, PDGF-B gene transfer promotes the regeneration of tooth- supporting structures when adenovirus-encoded PDGF-B is applied to periodontal osseous defects (23). Another study examined the effect of gene transfer by adenovirus encoding PDGF-A and PDGF-B on a human gingival fibroblast repopulation in an ex vivo wound-healing model and found that gene expression was maintained for at least 10 d and that PDGF-B gene transfer stimulated an increase of more than four-fold in cell repopulation above that of PDGF-A and controls (24). Although adenoviral delivery of PDGF-B demonstrates a promising future in periodontal regeneration, little is known about its longterm effect on signal transduction in cells isolated from the periodontium. Human gingival fibroblasts are a possible cell target for periodontal tissue regeneration because of several advantages, such as easy accessibility, richness in cell resource and higher proliferation potential. In our previous work, we have already shown that human gingival fibroblasts can respond to adenoviralencoded platelet-derived growth factor-A (21) and can be used to stimulate periodontal soft tissue repair ex vivo (24). The present study sought to evaluate the effect of gene transfer of PDGF-B by adenovirus on human gingival fibroblasts and the subsequent regulation of genes and cell-surface proteins associated with cellular signaling.

Material and methods

Adenovirus construction

The construction of the adenovirus encoding *PDGF-1308* (the dominant

negative mutant of platelet-derived growth factor-A) has been previously described (25). The recombinant adenoviruses encoding human *PDGF-B* (Ad-PDGF-B) and luciferase (Ad-Luc) are driven by a cytomegalovirus promoter and were gifts from Tissue Repair Company (26), San Diego, CA, USA.

Cell culture and gene transduction

Primary human gingival fibroblasts used for this study were kindly gifted to us by Dr Martha Somerman (University of Washington, Seattle, WA, USA). Human gingival fibroblasts were isolated separately from gingival tissue biopsy samples from three healthy human patients. Low passage cells (passages 4-7) were cultured and maintained in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin/streptomycin and 2 mM glutamine, then plated in 60×15 -mm dishes. Confluent cultures were brought to a stage of quiescence by rinsing the monolayers with phosphate-buffered saline and reducing the serum concentration to 0.1% for 24 h prior to adenovirus transduction or treatment with recombinant human platelet-derived growth factor-BB (a kind gift of BioMimetic Therapeutics Inc., Franklin, TN, USA). For all experiments the cells were treated either with recombiadenoviruses (Ad-PDGF-B, nant Ad-PDGF-1308, Ad-Luc) for 2 h at a multiplicity of infection of 200 or with 20 ng/mL of recombinant human platelet-derived growth factor-BB. Cells were incubated for various periods of time and harvested at 1, 6, 12, 24, 48, 72 and 96 h.

[Methyl-³H]thymidine incorporation assay

Human gingival fibroblast cells were seeded in 12-well plates at a concentration of 2×10^4 cells per well. After different treatments (recombinant human platelet-derived growth factor-BB, Ad-PDGF-B, Ad-PDGF-1308 or no treatment), cells were cultured with serum-free Dulbecco's modified Eagle's minimal essential medium. Two days later, 2×10^5 counts per minute of [methyl-³H]thymidine was added to each well. On day 7, the medium was removed and each well was washed twice with cold phosphate-buffered saline. The DNA in each well was precipitated with 5% cold trichloroacetic acid at 4°C for 2 h, solubilized with 1% sodium dodecyl sulfate solution at 55°C for 2 h and then the radioactivity of [methyl-³H]thymidine in the solution was counted using a scintillation counter (Wallac 1410; Perkin-Elmer, Waltham, MA, USA).

Western blot analysis

Whole-cell lysates were obtained from transduced human gingival fibroblast cells by washing cell monolayers twice with ice-cold phosphate-buffered saline, adding 250 µL of ice-cold cell lysis buffer (Bio-Rad, Alameda, CA, USA) directly to each dish and harvesting using cell scrapers. The total protein concentration was determined using the Bio-Rad Protein Assay with measurement of the absorbance at 595 nm. After heat denaturation (95°C, 5 min), 10 µg of total protein from each sample was added to 5× sample buffer (0.06 м Tris, pH 6.8, 2% sodium dodecyl sulfate, 0.3 м β-mercaptoethanol, 0.1% bromophenol blue, 10% glycerol) and the samples were electrophoresed (80 V, 2 h) through 10% acrylamide gels in Tris-glycine-sodium dodecyl sulfate running buffer. Protein samples were then transferred to immunoblot poly(vinylidene difluoride) membranes (Bio-Rad) in transfer buffer, pH 8.3 (25 mM Tris, 192 mM glycine, 20% methanol) for 90 min at 400 mA in a 4°C cold room. The membrane was then incubated in TTBS (100 mM Tris, 0.9% NaCl, 0.5% Tween 20) containing 5% nonfat dry milk for 1 h to block nonspecific binding. The blocking solution was discarded and the membrane was incubated overnight in fresh blocking solution containing diluted primary antibodies. The membranes were then washed with TTBS for 15 min, three times, then incubated in fresh blocking solution containing a 1:3000 dilution of anti-rabbit horseradish peroxidase (Amersham Corp. Piscataway, NJ, USA) for 1 h. The membranes were washed as described above and prepared for chemiluminescent detection by immersing the blot in a 1:1 mixture of reagents from an ECL Detection Kit (Amersham Corp.), then exposed to Biomax MS film (Kodak, Rochester, NY, USA) for 30-120 s before development. Densitometric scanning (Bio-Rad Molecular Imager ChemiDoc XRS System; Bio-Rad) was performed by normalizing platelet-derived growth factor receptor expression to glyceraldehyde-3-phosphate dehydrogenase for each of the treated primary cell cultures from three different human biopsies to ascertain receptor expression patterns following gene or protein delivery. Antibodies used in the present study were: rabbit anti-human plateletderived growth factor-a receptor polyclonal antibody and rabbit anti-human platelet-derived growth factor-ß receptor polyclonal antibody (Upstate Cell Signaling, Charlottesville, VA, USA), rabbit polyclonal anti-phospho-Akt (Ser473), rabbit polyclonal anti-Akt, rabbit polyclonal anti-GAPDH (all from Cell Signaling Technology, Danvers, MA, USA). In order to detect the secreted platelet-derived growth factor-BB, cell culture medium was collected on day 4, followed by dialysis in Amicon Ultra spin columns (Millipore, Billerica, MA, USA) and lyophilization. Then, the samples were reconstituted in H₂O and loaded for western blot analysis. Rabbit polyclonal antiplatelet-derived growth factor-B (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to detect platelet-derived growth factor-B expression.

Reverse transcription-polymerase chain reaction

Total RNA was extracted from human gingival fibroblast cells using the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA, USA). Approximately 1 μ g of RNA was reverse transcribed in a 20- μ L reverse transcription reaction using a Retroscript kit (Ambion, Austin, TX, USA). cDNA was generated using random primers. Ten micro-liters of the cDNA was used as a template for polymerase chain reaction

amplification to detect *PDGF-B* as well as the housekeeping gene, *GAPDH*.

Proteomic array for phosphoproteins

In order to assess the relative level of phosphorylation in a given sample, a human Phospho-MAPK array kit (R&D Systems, Minneaplolis, MN, USA) was utilized to measure simultaneously the phosphorylation levels of 23 different mitogen-activated protein kinases (MAPKs) and other serine/ threonine kinases. Each array contains duplicate validated control and capture antibodies for specific kinases. The MAPK array was performed according to the manufacturer's protocol. Briefly, human gingival fibroblast cells that had undergone the different experimental treatments were lysed using the lysis buffer in the kit. Array membranes were blocked at room temperature for 1 h and were then incubated with 800 µg of total cell lysate overnight at 4°C. The next day, after washing three times, array membranes were incubated with a phospho-site specific biotinylated antibody cocktail for 2 h at room temperature. The membranes were washed a further three times, developed using the ECL Detection Kit (Amersham), then exposed to Biomax MS film (Kodak).

Results

Adenoviral gene delivery of *PDGF-B* results in sustained expression of platelet-derived growth factor-BB in human gingival fibroblast cells

We first assessed the expression of exogenous PDGF-B in primary human gingival fibroblasts by adenoviral gene delivery. In our previous study, using green fluorescent protein reporter protein, we showed that adenoviral gene delivery has a high transduction efficiency in fibroblasts (> 95% of dermal fibroblasts were transduced) (22). Similarly, in the present study we detected the transcription of exogenous PDGF-B 6 h after exposure to Ad-PDGF-B, and transcription was sustained for at least 96 h (Fig. 1A). This effect was not observed after transfection of either the Ad-Luc con-



Fig. 1. Adenoviral gene delivery of PDGF-B results in the sustained expression of plateletderived growth factor-B in human gingival fibroblast cells. (A) Human gingival fibroblast cells were cultured to confluence and were then transferred to serum-free medium containing Ad-PDGF-B (multiplicity of infection = 200), Ad-Luc, Ad-PDGF-1308, or platelet-derived growth factor-BB protein (20 ng/mL). The cells were then allowed to incubate for 2-3 h before removal and replacement with serum-free medium. The cells were then harvested for RNA extraction at 6, 12, 24, 48, 72 and 96 h post-infection. Approximately 1.0 µg of total RNA was then used to generate cDNA with oligo dT primers. The resulting cDNA was used as a template for polymerase chain reaction amplification using human gene-specific primers for PDGF-B or GAPDH. The results show that only the treatment with Ad-PDGF-B caused a measurable increase in PDGF-B gene expression. The results obtained following treatment with Ad-Luc, Ad-PDGF-1308 or platelet-derived growth factor-BB protein showed no detectable expression of the *PDGF-B* gene (data not shown). (B) Approximately 2×10^5 cells were seeded in 100-cm dishes. After transient infection with Ad-PDGF-1308 or Ad-PDGF-B, the cells were cultured in serum-free medium. Conditioned media were collected at 96 h. After dialysis and lyophilization, samples were reconstituted in H₂O, loaded for non-reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and detected using anti-PDGF-BB. Our Ad-PDGF-B vector encodes a 160 amino acid platelet-derived growth factor-B isoform with a molecular weight of 18.1 kDa; therefore, a dimer in the non-reduced SDS gel was about 36.2 kDa. For the recombinant human platelet-derived growth factor-BB control, the dimer of the 109 amino acid isoform displayed a molecular weight of 25 kDa. hGAPDH, human glyceraldehyde-3-phosphate dehydrogenase; PDGF-B, platelet-derived growth factor-B; rhPDGF-BB, recombinant human platelet-derived growth factor-BB.

trol (Fig. 1A) or Ad-PDGF-1308 (data not shown). Furthermore, exogenously expressed *PDGF-B* was translated and secreted into cell culture medium as a dimer, which was detected by western blot analysis 4 d after treatment with Ad-PDGF-B but not after treatment with Ad-PDGF-1308 (Fig. 1B). Our Ad-PDGF-B vector encodes a 160 amino acid platelet-derived growth factor-B isoform with a molecular weight of 18.1 kDa; therefore, in nonreducing conditions, this results in a dimer of about 36.2 kDa. For the recombinant human platelet-derived growth factor-BB control, the dimer of the 109 amino acid isoform displayed a molecular weight of 25 kDa.

Adenoviral gene delivery of *PDGF-B* maintains human gingival fibroblast proliferation

Next, we sought to determine whether the exogenous expression of *PDGF-B* was biologically active. After treatment with Ad-PDGF-B, human gingival fibroblasts tended to form greater cell polarization distinct from that observed following treatment with recombinant human platelet-derived growth factor-BB or with the Ad-PDGF-1308 control (Fig. 2A). Compared to treatment with Ad-PDGF-1308 or transient recombinant human platelet-derived growth factor-BB, Ad-PDGF-B administration maintained the proliferation of human gingival fibroblasts in serum-free conditions and stimulated robust DNA synthesis, as shown by the [methyl-³H]thymidine-incorporation assay (Fig 2B).

Gene delivery of Ad-PDGF-B prolonged downregulation of the growth arrest-related gene $PDGF\alpha R$ and of $PDGF\beta R$

In the condition of serum deprivation, fibroblasts remain in the state of growth arrest (G_0) , which is associated with increased expression of the gas gene *PDGF* αR as well as of *PDGF* βR . We examined the impact of Ad-PDGF-B transduction on the levels of plateletderived growth factor-a receptor and platelet-derived growth factor-ß receptor. The steady-state levels of plateletderived growth factor-a receptor and platelet-derived growth factor-ß receptor for the Ad-PDGF-1308-treated human gingival fibroblasts remained at similar levels over the 96-h observation period. Treatment of human gingival fibroblasts with recombinant human platelet-derived growth factor-BB protein elicited a reduction in the levels of platelet-derived growth factor-a receptor and platelet-derived growth factor-B receptor within 1 h and these levels remained low for 12 h before returning to normal baseline levels by 24 h (Fig 3). By contrast, human gingival fibroblast cells treated with Ad-PDGF-**B** (multiplicity of infection = 200) showed a delayed, but prolonged and greater, reduction in both plateletderived growth factor- α and - β receptor protein levels starting at about 24 h, with the levels remaining reduced for at least 96 h (Fig. 3).

Proteomic array to determine phosphorylation of intracellular proteins

In these experiments our goal was to identify the effects on signal transduc-



Fig. 2. (A) Cells were treated as described in the legend to Fig. 1 and photographed at 72 h. Compared to treatment with control (serum-free), Ad-PDGF-1308, or recombinant human platelet-derived growth factor-BB, human gingival fibroblasts transduced with Ad-PDGF-B resulted in colony-like growth, as indicated by the arrows (20× magnification). (B) Human gingival fibroblast cells were seeded in 12-well plates at a concentration of 2×10^4 cells per well. After different treatments (recombinant human platelet-derived growth factor-BB, Ad-PDGF-B, or Ad-PDGF-1308), the cells were cultured with serum-free Dulbecco's modified Eagle's minimal essential medium without medium change during the experiment. Two days later, 2×10^5 counts per minute of [methyl-³H]thymidine was added to each well. On day 7, the medium was removed and each well was washed twice with cold phosphate-buffered saline. The DNA in each well was precipitated with 5% cold trichloroacetic acid at 4°C for 2 h, solubilized with 1% sodium dodecyl sulfate solution at 55°C for 2 h, then the radioactivity of [methyl-³H]thymidine in the solution was counted using a scintillation counter. Ad-PDGF-B stimulated robust DNA synthesis when compared with the other treatments. NS, no significant difference; (n = 6 samples).

tion elicited by Ad-PDGF-B gene delivery. After recombinant human platelet-derived growth factor-BB binds to tyrosine receptor kinases, it triggers a cascade of downstream signal transduction pathways, in which kinase activation plays a central role. Because we observed (by western blotting) prolonged downregulation of plateletderived growth factor- α and - β receptor protein levels following treatment with Ad-PDGF-B, we wanted to determine whether adenoviral delivery would also extend intracellular signaling events. Therefore, we examined the phosphorylation state of numerous kinases by using a human phospho-MAPK array that contains 21 different specific intracellular kinase antibodies (Table 1). This array allowed the simultaneous relative quantification of the phosphorylation state of MAPKs in three major families (ERK1/2, JNK1–3 and p38), and also detected another nine important intracellular tyrosine/serine kinases, including Akt, GSK-3 and p70 S6 kinase, in the same cell lysate



Fig. 3. Gene delivery of Ad-PDGF-B prolongs the downregulation of the growth arrest-related gene (*gas*) *PDGF* α *R* as well as *PDGF* β *R*. Western blot was used to analyze platelet-derived growth factor- α receptor and platelet-derived growth factor- β expression levels in human gingival fibroblasts in response to treatment with either recombinant human platelet-derived growth factor-BB (20 ng/mL), or Ad-PDGF-B. Human gingival fibroblasts from three individuals were tested, with representative data shown here. Human gingival fibroblasts were exposed to Ad-PDGF-1308 (multiplicity of infection = 200), Ad-PDGF-B (multiplicity of infection = 200) or 20 ng/mL of recombinant human platelet-derived growth factor-BB in serum-free medium for 2 h. Then, the medium was removed and human gingival fibroblasts were maintained in serum-free medium for 1, 6, 12, 24, 48, 72 and 96 h. Cells were lysed and 30 µg of total protein from cell lysates was subjected to western blotting using antibodies to platelet-derived growth factor- α receptor and platelet-derived growth factor- β receptor. The blots were then stripped and reprobed with anti-GAPDH to determine the relative loading of protein in each lane. Densitometric scanning was performed by normalizing platelet-derived growth factor receptor expression to glyceraldehyde-3-phosphate dehydrogenase for the treated primary cell cultures from three different human biopsies. The graphs demonstrate the cumulative results from three individuals with the blots showing the typical result. The graphs show that treatment with Ad-PDGF-B resulted in sustained downregulation of platelet-derived growth factor- α receptor and platelet-derived growth factor- α receptor for up to 96 h. GAPDH, glyceral

Table 1. Human phosphoproteins simultaneously measured the phosphorylation levels of 21 different mitogen-activated protein kinases (MAPKs) and other serine/threonine kinases following *PDGF-B* gene therapy

MAPK family	ERK1, ERK2
	JNK1, JNK2, JNK3, JNK pan
	p38α, p38β, p38γ, p38δ
Others	Akt1, Akt2, Akt3, Akt pan, RSK1, RSK2, MSK2,
	GSK-3α/β, GSK-3β, HSP27, p70 S6 kinase

(Table 1). With this array we focused on phophorylation patterns at early stage (1 h) and late-stage (72 h and 96 h) time-points. Akt1, Akt2 and Akt3 were the most sensitive intracellular signal markers to platelet-derived growth factor treatment of all the 21 kinases that were tested. Our results showed that Ad-PDGF-B treatment revealed a similar, but delayed, phosphorylation pattern as that of recombinant human plateletderived growth factor-BB: significant Akt phosphorylation was seen by 1 h after stimulation of human gingival fibroblasts by recombinant human platelet-derived growth factor-BB, but the effect disappeared by 72 h; exposure

to Ad-PDGF-B resulted in Akt phosphorylation at the late stage, which can be seen from arrays at both 72 and 96 h (Fig. 4). Unstimulated human gingival fibroblast lysates demonstrated very low basal levels of phosphorylation in all the tested kinases under serumdeprivation conditions, and this was also found for the Ad-PDGF-1308 control.

Sustained phosphorylation of Akt in adenoviral gene delivery

Based on our findings from the proteomics array, Akt was further evaluated as a key intracellular molecule that mediates the biological function of adenoviral PDGF gene delivery. These experiments were designed to explore in further detail the time curve of Akt activation during Ad-PDGF-B transduction, compared with exposure to recombinant human platelet-derived growth factor-BB. Our results showed that in cells exposed to recombinant human platelet-derived growth factor-BB, phosphorylation of Akt, which peaked at 1-6 h, was sustained only for 12-24 h. In human gingival fibroblasts treated with Ad-PDGF-B, the phosphorylation of Akt was initially seen as early as 6 h post-transduction and remained elevated for at least 96 h, to a greater relative extent than plateletderived growth factor-BB (Fig. 5). Following Ad-PDGF-1308 delivery, human gingival fibroblasts did not show any Akt phosphorylation. We also examined the phosphorylation of Erk1/ 2 in different treatments. Ad-PDGF-B transduction elicited modest increases of Erk1/2 phosphorylation at the later time-points of 48-96 h, a result that was



Fig. 4. Proteomic array to identify intracellular phosphorylation proteins that mediate either immediate or long-term effects of protein or gene delivery. The human phospho-MAPK array kit was utilized to measure simultaneously the phosphorylation levels of target kinases. Human gingival fibroblast cells treated with 20 ng/mL of recombinant human platelet- derived growth factor-BB, Ad-PDGF-B (multiplicity of infection = 200), Ad-PDGF-1308 (multiplicity of infection = 200) or serum-free medium were harvested at 1, 72 and 96 h post-treatment. After blocking, 800 μ g of human gingival fibroblast cell lysates were incubated with membranes at 4°C overnight. Each membrane contained duplicates, validated controls and capture antibodies for specific kinases. After washing, a phospho-site-specific biotinylated antibody cocktail was added the following day. Each array was identically treated with detection reagent and film. Treatment with Ad-PDGF-B resulted in the phosphorylation of Akt in human gingival fibroblast cells at the late stage, which is similar to the immediate response to recombinant human platelet-derived growth factor-BB protein. 1, Akt1; 2, Akt2; 3, Akt3; 4, pan-Akt; Ctrl, control.



Fig. 5. Sustained phosphorylation of Akt in adenoviral gene delivery. Western blotting was used to examine the time curve of Akt phosphorylation after gene delivery. Human gingival fibroblasts from three patients were tested and representative data are shown here. Human gingival fibroblast cells were exposed to Ad-PDGF-1308 (multiplicity of infection = 200), Ad-PDGF-B (multiplicity of infection = 200) or 20 ng/mL of recombinant human plateletderived growth factor-BB in serum-free medium for 2 h. Then, cell lysates at 1, 6, 12, 24, 48, 72 and 96 h were subjected to western blotting using antibodies to phosphor-Akt and pan-Akt. The graphs show the relative ratio of phosphorylated Akt levels normalized to total Akt (analyzed by densitometric scanning). Recombinant human platelet-derived growth factor-BB induced transient Akt phosphorylation, which lasted about 24 h. However, with Ad-PDGF-B transduction, the phosphorylation of Akt was first seen as early as 6 h post-infection, and remained elevated for at least 96 h to a greater relative extent.

consistent with our proteomic array data (data not shown).

Discussion

A major goal of this study was to gain knowledge of the molecular mecha-

nisms by which adenoviral gene delivery of *PDGF-B* mediates periodontal tissue regeneration. Our previous work showed that tooth-supporting structures could be re-engineered by Ad-PDGF-B. By using human gingival fibroblast cells, we also demonstrated

that platelet-derived growth factor gene transfer has a potential use in periodontal soft tissue-engineering applications (24). Although the use of Ad-PDGF-B has enabled several advancements to be made in the promotion of periodontal regeneration, little is known about its effects in signal transduction from cells isolated from the periodontium. This study examined some of the cellular signaling events within human gingival fibroblasts resulting from adenoviral gene delivery of PDGF-B compared with direct protein delivery. This information aids in an improved understanding of these pathways that are likely to be critical in controlling the clinical outcomes of periodontal regenerative therapies.

Adenoviral vectors are known to be an effective tool for delivering genes to a broad range of cell types and tissues (27). Also, adenovirus has a high transduction efficiency and does not integrate into the host genome of the transduced cells (28). Compared with the short half-life of recombinant platelet-derived growth factor protein, our previous work showed that adenoviral gene delivery to rat periodontal sites induced marker gene expression, which was maintained at a high level at least until day 4 and rapidly decreased to 20% by day 14 (23). Therefore, we believe that by establishing the changes in signaling molecules in the first 96 h after treatment with Ad-PDGF-B we can understand its extended effects in more detail. We first examined the transduction efficiency of PDGF by Ad-PDGF-B gene transfer to human gingival fibroblasts using reverse transcription-polymerase chain reaction and observed a high level of PDGF gene expression for at least 96 h, and evident as early as 6 h post-infection (Fig 1). This correlates well with previous work by our group and by others, which consistently showed a sustained production of plateletderived growth factors delivered by adenovirus (21,24,29) within other cells from periodontia. In view of the fact that some of the platelet-derived growth factor-BB protein may adhere to the polymer surface because of strong negative charges, we would expect the yield of platelet-derived

growth factor-BB protein from the Ad-PDGF-B-transduced human gingival fibroblasts to be in the order of ~ 40 ng of protein per 10⁶ cells. This was effective to maintain cell proliferation in serum-free medium (Fig. 2B). Also it would provide an efficacious dose of the growth factor in vivo for periodontal wound healing applications and could abrogate the need for continuous delivery compared with the topical 'pulse' administration of platelet-derived growth factor-BB protein that typically results in the transient (< 24 h) release of platelet-derived growth factor-BB in vivo (16).

Platelet-derived growth factor is a soluble protein that stimulates cell proliferation and migration through membrane receptors of the receptor tyrosine kinase family. Platelet-derived growth factor-A only binds to plateletderived growth factor- α receptor, whereas platelet-derived growth factor-B can bind to both α and β receptors. Platelet-derived growth factor binding to the receptors also activates a variety of intracellular signaling molecules (3) and the platelet-derived growth factor- α and $-\beta$ receptors activate many overlapping signaling pathways. The activated receptors phosphorylate a large number of substrates (more than 20), including themselves, which initiates a complex network of signaling cascades. Activation of either the α or the β receptor can stimulate cell migration, although platelet-derived growth factor-B is generally a stronger inducer than platelet-derived growth factor-A (30-32). In addition to their traditional role as receptor tyrosine kinases, platelet-derived growth factor- α receptor and platelet-derived growth factor-ß receptor have been associated with cell growth arrest (17). Lih et al. showed that platelet-derived growth factor- α receptor was highly expressed during the cell growth arrest stage induced by serum deprivation (33). Vaziri et al. reported that serum deprivation induced growth arrest in G₀ and is associated with increased expression of platelet-derived growth factor- β receptor (34). We previously found that Ad-PDGF-A gene transfer resulted in sustained activation and subsequent downregulation of the

platelet-derived growth factor-a receptor, a direct result of continued signaling by ligand binding (22). Vaziri et al. also found that the adenovirus continuously produced platelet-derived growth factor-AA dimers and that prolonged platelet-derived growth factor expression activated and downregulated platelet-derived growth factor-a receptor for an extended period of time. Carmen et al. reported that treatment with recombinant human platelet-derived growth factor-BB induced the downregulation of platelet-derived growth factor receptor in mouse hepatic stellate cells (35). In the present study we measured a similar downregulation of both α and β platelet-derived growth factor receptors by Ad-PDGF-B gene transfer within human gingival fibroblast cells. The kinetics of platelet-derived growth factor- α and platelet-derived growth factor- β receptor protein expression in human gingival fibroblast cells were nearly identical: downregulated after 6 h post-transduction and maintained at very low levels for at least 96 h (Fig 3).

Protein phosphorylation is a fundamental mechanism for the regulation of many aspects of cellular functions. One of the important roles of protein phosphorylation is in signal transduction pathways, which are regulated at many levels, from the activation of cellsurface receptors to the stimulation of transcription factors. Detection of the phosphorylation state of intracellular proteins would provide valuable information on the activation state of human gingival fibroblasts following the long-term delivery of plateletderived growth factor. In the present study, by using a human phospho-MAPK array, we simultaneously assessed the phosphorylation of 23 different intracellular kinases, including three major members of the MAPK family (ERK1/2, JNK1-3 and p38), Akt, GSK-3 and p70 S6 kinase. Our results from the protein array (Fig. 4), which were confirmed by western blotting, showed that Akt activation may play an important role by mediating the effect of long-term plateletderived growth factor delivery in human gingival fibroblasts. In our

experiment, adenovirus was delivered to human gingival fibroblasts by transient transfection (only 2 h), which explains in part why the phosphorylation levels of Akt at later time-points were relatively low. Akt (also known as protein kinase B), functions as a downstream target of phosphatidylinositol-3-kinase and plays a critical role in controlling cell survival and apoptosis (36,37). Akt is inactive in serum-starved conditions, but is activated by various growth factors or including platelet-derived stimuli. growth factor (25). It is a general survival signal mediator, which inhibits apoptosis by phosphorylating or inactivating several downstream targets, such as nuclear factor-kB, Bad, forkhead transcription factors and caspase-9 (38). Thus, the sustained activation of Akt after adenoviral platelet-derived growth factor-B delivery in human gingival fibroblasts helps us to understand, in more detail, a mechanism by which PDGF gene therapy supports tissue repair (24). This is also supported by the work of Mangi et al., which showed that mesenchymal stem cells transduced with Akt1 can repair infarcted myocardium, probably via promoting cell survival after cell transplantation (39). Recently, Akt was also found to regulate the growth arrest-related genes $PDGF\alpha R$ and PDGFBR. Activation of Akt inhibits the expression of $PDGF\alpha R$ and PDGFβR via activating mTOR. This suppression probably occurs as a result of decreased transcription of PDGFR and/or reduced mRNA half-life (40,41). Therefore, the sustained decrease of the platelet-derived growth factor- α and - β receptors (Fig. 3) could be partly caused by the prolonged activation of Akt.

In conclusion, this study demonstrated a high efficiency of *PDGF-B* gene transfer into human gingival fibroblasts using adenovirus, with expression of the *PDGF* gene detected up to 96 h post-infection. This has promise for clinical applications as direct application of recombinant human platelet-derived growth factor-**BB** for periodontal wound healing applications has proven effective yet typically displays a much shorter halflife. Our data also shows that specific intracellular signaling pathways (i.e., those involving platelet-derived growth factor receptors α and β , and Akt) mediate unique responses to growth factors via gene delivery within human gingival fibroblasts. These data on *PDGF* gene delivery contribute to an improved understanding of these pathways that are likely to play a role in the control of clinical outcomes of periodontal regenerative therapy.

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