Recombinant Human Platelet-Derived Growth Factor BB (rhPDGF-BB) and Beta-Tricalcium Phosphate/Collagen Matrix Enhance Fracture Healing in a Diabetic Rat Model

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ABSTRACT: Diabetes mellitus is a common systemic disease that has been associated with poor fracture healing outcomes. The mechanism through which diabetes impairs bone regeneration is unknown. One possible mechanism may be related to either decreased or uncoordinated release of local growth factors at the fracture site. Indeed, previous studies have found reduced platelet-derived growth factor (PDGF) levels in the fracture callus of diabetic rats, suggesting that local application of PDGF may overcome the negative effects of diabetes and promote fracture healing. To test this hypothesis, low (22 μg) and high (75 μg) doses of recombinant human PDGF-BB (rhPDGF-BB) were applied directly to femur fracture sites in BB Wistar diabetic rats that were then compared to untreated or vehicle-treated animals. rhPDGF-BB treatment significantly increased early callus peak torque values (p < 0.05) at 8 weeks after fracture as compared to controls. High dose rhPDGF-BB treatment increased callus bone area at 12 weeks postfracture. These data indicate that rhPDGF-BB treatment ameliorates the effects of diabetes on fracture healing by promoting early cellular proliferation that ultimately leads to more bone formation. Local application of rhPDGF-BB may be a new therapeutic approach to treat diabetes-impaired fracture healing. © 2009 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res

Keywords: diabetes mellitus; fracture; BB Wistar rat; rhPDGF-BB; drug delivery

Twenty million Americans have diabetes mellitus (diabetes) and 500,000 to 1,000,000 new cases are diagnosed each year. The prevalence of diabetes is estimated to exceed 30 million patients in the United States by 2010.1

The association between diabetes and impaired osseous healing has been documented in clinical and experimental settings.2–5 Several clinical studies have evaluated complications following elective arthrodesis in diabetes patients and noted a significant incidence of delayed union, nonunion, and pseudoarthrosis.4 Furthermore, chemically induced and spontaneous diabetic animal models also have demonstrated impairments in fracture healing.3–8 In various fracture healing models, diabetes correlated with a reduction in fracture callus cellular proliferation, collagen synthesis, and biomechanical properties.2,7,9,10

The mechanism by which diabetes impairs fracture healing is unknown. In normal fracture healing a blood clot forms at the fracture site that entraps platelets within the fibrin matrix. The platelet α-granules act as a reservoir of critical early growth factors.11 Degranulation of the α-granules releases multiple growth factors, including platelet derived growth factor (PDGF) and transforming growth factor β (TGFB-β), into the fracture site. Previous studies in diabetic rats, however, have demonstrated that at early time points after fracture (2, 4, and 7 days), levels of PDGF, TGFB-β, insulin-like growth factor I (IGF-I), and vascular endothelial growth factor (VEGF) are reduced,6,8 and correspondingly cellular proliferation is decreased at the fracture site. These observations suggest that diabetes-impaired fracture healing occurs secondarily to a deficiency in an early phase of bone healing, potentially as a result of either inadequate biological signals or a discordant growth factor signaling cascade. The combined outcome results in impaired proliferation and differentiation of mesenchymal cells to osteoblasts as well as decreased angiogenesis. Consequently, therapeutic options that include PDGF should be investigated for their ability to reverse compromised fracture healing in diabetes patients.

PDGF is a family of growth factors that contain five different members that are found naturally in the body. They can form a series of disulfide-linked homo and hetero dimers including PDGF-AA, AB, BB, CC, and DD.12,13 PDGF works by binding to two distinct cell-surface receptors present on cells of mesenchymal origin. PDGF is released from platelets and macrophages during the earliest phase of fracture healing,14 and initiates a series of events as a result of its chemotactic and mitogenic properties. PDGF functions in a macrophage autocrine feedback loop to stimulate the synthesis and release of growth factors and cytokines.15 PDGF also stimulates a variety of mesenchymal-derived cell types to enhance DNA synthesis, increase collagen deposition, and stimulate synthesis of extracellular matrix.16 In synergy with stromal-derived factor-1 (SDF-1), PDGF-BB promotes bone marrow cell growth and resistance to α-interferon-2α-induced apoptosis,17 suggesting that PDGF and SDF-1 may act in synergy in promoting the self-renewal and survival capacity of bone marrow stromal stem cells.

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In vitro, PDGF has little or no effect on osteoprogenitor cell alkaline phosphatase activity. However, PDGF stimulates osteoblast type I collagen and osteopontin expression in vitro and osteopontin expression in vivo. PDGF-AB and -BB are the prevalent circulating isoforms, whereas normal unstimulated cells of the osteoblast lineage synthesize primarily PDGF-AA. PDGF-BB is more potent than PDGF-AB or PDGF-AA, although all three isoforms have mitogenic activity in cultures of osteoblast-enriched cells from fetal rat calvariae. Rydziel et al. demonstrated that PDGF-BB can increase PDGF-A mRNA expression and PDGF-AA synthesis in osteoblast-enriched cultures of 22-day-old fetal rat calvariae.

A model of diabetes-impaired fracture healing is the diabetic-prone BB Wistar rat, which develops insulin-dependent diabetes mellitus through a spontaneous, autoimmune destruction of the pancreatic beta cells, which closely resembles human type I diabetes. Approximately 50% of the diabetic-prone rats develop glycosuria by 85 days of age. If not treated with insulin, the rats will die within 5–10 days after the onset of glycosuria.

In the current study, the diabetic BB Wistar rat was used to test the hypothesis that local delivery of recombinant human PDGF BB homodimer (rhPDGF-BB) would mitigate against compromised healing in a diabetic fracture model. The expectation was that the application of rhPDGF-BB at the fracture site would increase early cellular proliferation resulting in enhancement of impaired fracture healing.

METHODS

Animal Model

The diabetic-prone BB Wistar rats used in the study were either purchased at 60 days of age from Biomedical Research Models (BMR, Worcester, MA) or were obtained from a breeding colony at UMDNJ-New Jersey Medical School (NJMS). The breeding colonies at BMR and UMDNJ were established from diabetic-prone BB Wistar rats originally obtained from BioBreeding (Toronto, Canada) and were approximately 75 generations apart. Animals from both facilities were considered equivalent for these experiments.

Only diabetic-prone BB Wistar rats that had become diabetic and that were treated with insulin implants as described below were used in this study. The diabetic-prone BB Wistar rats were checked for glycosuria approximately three times a week. Once glycosuria was detected, blood glucose levels were measured using tail vein blood and an Accu-Chek ADVANTAGE glucometer (Boehringer Mannheim Corp., Indianapolis, IN). When blood glucose levels exceeded 250 mg/dL, the rats were treated with a slow-release insulin implant (LINPLANT, Canada) placed subcutaneously in the dorsal neck. The insulin implant provides approximately 30 days of constant insulin release. The amount of insulin implant used was adjusted to maintain blood glucose levels between approximately 300 and 500 mg/dL, which represents a poorly controlled state of diabetes with glycosuria, but no sign of ketonuria. At euthanasia, 2 mL of whole blood was collected and glycosylated hemoglobin (HbA1c) levels were determined using an HbA1c kit (Diazyme Laboratories, Poway, CA). HbA1c is a time-averaged measure of blood glucose control and can be twice as high in patients with poor glucose control when compared to normal patients. A total of 110 animals were used to measure cellular proliferation (n = 26), histomorphometric (n = 18), and biomechanical (n = 66) parameters of fracture healing. All research protocols were approved by the Institutional Animal Care and Use Committee at the University of Medicine and Dentistry of New Jersey.

Fracture Model

Fourteen days after the onset of diabetes, a closed mid-diaphyseal fracture was made in the right femur as described previously. Following fracture, a lateral incision was made above the fracture site and the underlying muscle bluntly dissected to expose the fracture. Matrix combined with rhPDGF-BB was then applied directly to the fracture site with minimal perturbation of the periosteum. Only rats that exhibited noncomminuted, transverse fractures, confirmed by radiographs, were used in this study.

Delivery System

Test material was delivered directly to the fracture site using a β-tricalcium phosphate/Type I bovine collagen matrix (80:20 v/v) (Kensey Nash Corp., Exton, PA). The β-TCP/collagen matrix was combined with buffer (20 mM sodium acetate, pH 6.0) or two different doses of rhPDGF-BB in acetate buffer (0.3 and 1.0 mg/mL; BioMimetic Therapeutics, Inc., Franklin, TN) to form a “puttylike” formulation. Approximately 0.2 g of β-TCP/collagen matrix was mixed with 600 μL of buffer or rhPDGF-BB solution and 100 μL of this formulation was applied directly around the fracture site. There were four experimental groups:

1. No treatment group: fracture with an intramedullary (IM) rod only.
2. Buffer group: fracture with IM rod plus β-TCP/collagen matrix combined with buffer.
3. Low dose group: fracture with IM rod plus β-TCP/collagen matrix combined with approximately 22 μg of rhPDGF-BB.
4. High dose group: fracture with IM rod plus β-TCP/collagen matrix combined with approximately 75 μg rhPDGF-BB.

Cellular Proliferation

Rats were injected intraperitoneally with 30 mg/kg of 5-bromo-2’-deoxyuridine (BrdU) (Sigma Chemical Co., St. Louis, MO) 1 h prior to euthanasia on day 4 postfracture. Standard techniques were used to prepare and stain tissue slides as described previously. Following decalcification with Immunoalkaline decalifier (Decal Corp., Tallman, NY), samples were embedded in paraffin and 5 μm thick, midsagittal sections through the femur were prepared. Cells positive for BrdU incorporation were detected by immunohistochemistry using commercially available reagents (DAKO Corp., Carpentaria, CA). Digital images of each fracture were collected with a microscope (BH2; Olympus) equipped with a camera (DXM1200F; Nikon). For each specimen, callus area was measured and BrdU-positive cells in the external callus were counted (Image Pro Plus-version 5.0; Media Cybernetics, Silver Spring, MD). All BrdU-positive cells in the external callus to a maximum of 1 cm proximal and distal of the fracture site and 3 mm from the external surface of the femur were
counted. The number of BrdU-positive cells was normalized per unit area of callus and only one datum per rat (BrdU-positive cells per mm²) was used for the statistical analysis. This cell quantification methodology was used in previous studies.7,8,27,28

Mechanical Testing
At 6 and 8 weeks postfracture, fractured and contralateral femora were resected, cleaned of soft tissue, and the IM rod removed. The harvested bones were stored at −20°C until testing. Standard torsional mechanical testing was performed as described previously.7,8 The peak torque (Tₚₑᵃₚ), torsional rigidity (TR), shear modulus (SM), and maximum shear stress (SS) were determined through standard equations modeling each femur as a hollow ellipse.29

Histomorphometry
Fractured femora were harvested at 12 weeks postfracture and embedded in polymethylmethacrylate.30 Histological sections were prepared and analyzed as described previously.7,8

Statistical Analysis
Analysis of variance (ANOVA) was performed followed by Holm-Sidak posthoc tests to identify differences between treatment groups (SigmaStat 3.0; SPSS Inc., Chicago, IL). Significance was established at p ≤ 0.05. To compare the biomechanical parameters between different groups and time points, the data were normalized by dividing each fractured value by the value for the corresponding intact, contralateral femur. Normalization was used to minimize biological variability due to differences in age and weight among rats, as the onset of diabetes occurred anywhere from ages 60 to 120 days.

RESULTS
Animal Model: General Health
Animals in all four treatment groups gained weight at an apparent comparable rate (Table 1). No statistical differences were found for body weight, blood glucose levels, or percent HbA1c between treatment groups at the time points tested, indicating that local rhPDGF-BB treatment had no systematic effect on glucose metabolism (Tables 1 and 2).

rhPDGF-BB Increased Diabetic Callus
Cellular Proliferation
At day 4 postfracture, cellular proliferation in the callus region was significantly higher in the two rhPDGF-BB groups as compared to the control groups (Figs. 1 and 2). However, no difference in cellular proliferation was detected between the low and high dose rhPDGF-BB groups. No difference in cellular proliferation was detected between the control groups.

rhPDGF-BB Treatment Enhanced Diabetic Callus
Biomechanical Properties
At week 6 postfracture, no difference in callus biomechanical properties was found between the treatment groups. However, the low dose rhPDGF-BB group had higher percent torsional rigidity and shear modulus values compared to the other treatment groups that trended toward significance (p = 0.079 and 0.064, respectively; Fig. 3). At week 8 postfracture, the percent peak torque to failure for the low dose rhPDGF-BB group was significantly higher (p < 0.05) than the no treatment or buffer group values (Fig. 4).

Histomorphometric Analysis of rhPDGF-BB Treatment on Diabetic Fracture Healing
At 12 weeks postfracture, histology indicated increased bony bridging in both rhPDGF-BB groups while the control groups showed delayed healing characterized by the continued presence of cartilage and a greater proportion of fibrous tissue (Fig. 5).

Table 1. General Health: Body Weight

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight at Day of Sacrifice (g)</th>
<th>Percent Weight Change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 Days (n = 26)</td>
<td>6 Weeks (n = 32)</td>
</tr>
<tr>
<td>No treatment</td>
<td>255 ± 46</td>
<td>381 ± 24</td>
</tr>
<tr>
<td>Buffer</td>
<td>266 ± 38</td>
<td>391 ± 37</td>
</tr>
<tr>
<td>Low dose</td>
<td>275 ± 10</td>
<td>361 ± 38</td>
</tr>
<tr>
<td>High dose</td>
<td>260 ± 43</td>
<td>413 ± 50</td>
</tr>
</tbody>
</table>

The data represent average values ± standard deviation.

Table 2. General Health: Blood Glucose and %HbA1c

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood Glucose (mg/dL)*</th>
<th>%HbA1c†</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>429 ± 64</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>Buffer</td>
<td>435 ± 49</td>
<td>13 ± 5</td>
</tr>
<tr>
<td>Low dose</td>
<td>426 ± 64</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>High dose</td>
<td>413 ± 79</td>
<td>14 ± 5</td>
</tr>
</tbody>
</table>

The data represent average values ± standard deviation.

*Blood glucose data represent mean data from animals in each treatment group resulting from measurements taken from blood drawn two or three times per week per animal up to sacrifice. Data are the mean of all measurements across all time points of the study (4 days, 6 weeks, 8 weeks, and 12 weeks). †Data for percent HbA1c are based on measurements taken from blood drawn at sacrifice and include animals utilized in 6 and 8 week mechanical testing and 12 week histomorphometry analysis.
Histomorphometric analysis revealed no significant differences in gap callus area between the four treatment groups 12 weeks after fracture (Fig. 6A). However, the callus percent mineralized bone area increased significantly for the high dose rhPDGF-BB group compared to the other groups (Fig. 6B). No significant difference in percent bone area was found between the low dose rhPDGF-BB and control groups. No β-TCP was evident in any of the specimens, suggesting that the matrix was resorbed completely by 12 weeks after fracture. These results underscore a normal progression of fracture healing in the rhPDGF-BB-treated rats.

Moreover, no ectopic or abnormal bone formation was observed in any treatment group.

**DISCUSSION**

Diabetes has been demonstrated to impair the fracture healing process. In animal models, diabetes has been reported to cause a reduction in early cellular proliferation, a delay in chondrogenesis, and a decrease in the biomechanical properties of the fracture callus. The precise molecular mechanism by which diabetes impairs these fracture healing processes is unknown. Several mechanisms are possible.
Diabetic fracture healing studies suggest that reduced PDGF and basic fibroblast growth factor (bFGF) expression correlated with diminished fracture callus cell proliferation and mechanical properties. PDGF and bFGF are potent mitogens and have been reported to enhance the release of additional signaling factors from mesenchymal cells that are involved in chondrogenesis and bone formation. Other diabetic fracture healing studies have shown that reduced expression of critical transcription factors correlated with impaired bone healing. For example, in a model of intramembranous bone formation, diabetes impaired expression of Runx2 and Dlx5, which are osteoblast differentiation regulators. In turn, bone formation and Type I collagen and osteocalcin expression were reduced. Other studies have shown that diabetes leads to reduced fracture callus biomechanical properties consistent with impaired healing.

In the present study, we evaluated the dose-dependent effect of rhPDGF-BB delivered in a β-TCP/Type I bovine collagen matrix on fracture healing in the diabetic BB Wistar rat model.

The rationale for the two rhPDGF-BB concentrations used in this study was based upon previous preclinical and clinical studies of the use of rhPDGF-BB to promote bone healing. Using doses similar to those used in this study, rhPDGF-BB enhanced periodontal bone repair in rats and humans. In addition, rhPDGF-BB treatment at doses used in this study significantly enhanced fracture healing in aged, osteopenic rats. Fracture callus cell proliferation was significantly higher in the diabetic rats treated with rhPDGF-BB, indicating that rhPDGF-BB treatment can ameliorate the deficit in callus cell proliferation observed in diabetic animals (Figs. 1 and 2). Torsional mechanical testing also indicated that low dose rhPDGF-BB treatment significantly increased fracture callus peak torque 8 weeks after healing (Fig. 4). These findings support the hypothesis that rhPDGF-BB induced fracture callus cell proliferation and led to a subsequent increase in the mechanical properties of the fracture callus.
The findings of this study are supported by those reported by Hollinger et al., in which rhPDGF-BB was found to accelerate fracture healing in geriatric, osteoporotic rats. Diabetes also may affect bone remodeling in the later stages of fracture healing. For example, while histomorphometric analysis at 12 weeks postfracture showed no significant difference in callus area, a significant increase in percent mineralized area was observed in the calluses treated with high dose rhPDGF-BB as compared to low dose rhPDGF-BB or control groups. These data suggest that rhPDGF-BB treatment may have a long-term effect that enhances the later stages of fracture healing in diabetic animals.

High dose rhPDGF-BB treatment significantly increased early callus cell proliferation but had no effect later on callus mechanical properties. In contrast, low dose rhPDGF-BB increased early callus cell proliferation and later on callus biomechanical properties. We interpret these data to indicate that in this model, high dose rhPDGF-BB treatment may delay differentiation of mesenchymal cells into chondrocytes and osteoblasts by extending the proliferative phase.

Previous studies have reported potential mechanisms of action for PDGF as a stimulator of bone healing. PDGF enhances DNA synthesis, increases collagen deposition, and stimulates synthesis of extracellular matrix in wound repair. In vitro, PDGF stimulates Type I collagen mRNA and protein synthesis in osteoblasts and chondrocytes. Further, PDGF has chemotactic and proliferative properties while purportedly suppressing differentiation of osteoprogenitor cells.

An additional crucial wound healing property of rhPDGF-BB is the promotion of new blood vessel formation through up-regulation of VEGF and stabilization of new capillaries by mural cells. VEGF is expressed by proliferating osteoblasts and hypertrophic chondrocytes and is a key regulator of endochondral bone formation.

To the best of our knowledge, this study is the first to examine the effect of local delivery of rhPDGF-BB on fracture healing in diabetic animals. The data indicate...
that local rhPDGF-BB treatment significantly enhances fracture healing.

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