Effect of Targeted Delivery of Bone Morphogenetic Protein-2 on Bone Formation in Type 1 Diabetes

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Purpose: Bone formation and healing are diminished in experimental type 1 diabetes. The present study investigated whether controlled local release of recombinant human bone morphogenetic protein 2 (rhBMP-2) stimulates bone defect healing in diabetes as a consequence of its anabolic effects on bone. Materials and Methods: Bilateral experimental circular bone defects were created in the temporal bones of 64 BALB/cByJ mice. Defects were treated with acellular collagen sponge plus 0.4 or 1.8 μg of rhBMP-2 per defect, and untreated defects served as controls. The healing of the defects over a 14-day period in diabetic and nondiabetic mice was analyzed histomorphometrically. Results: Diabetes inhibited bone formation in both untreated and BMP-treated bone defects. Controlled local release of rhBMP-2 significantly stimulated bone formation in diabetic animals, bringing it nearly to normal levels, and enhanced bone regeneration in normal animals. Conclusion: Recombinant human BMP-2 may be beneficial in treating deficient intramembranous bone formation in diabetes. Int J Oral Maxillofac Implants 2015;30:707–714. doi: 10.11607/jomi.3957

Key words: bone regeneration, diabetes, recombinant human bone morphogenetic protein-2

Undisturbed bone formation and regeneration are fundamental aspects of implant dentistry. Successful osseointegration of metallic implants in native or regenerated bone is intrinsically dependent on normal bone formation.1 Diabetes mellitus is classified as a risk factor for implant treatment, and severe or poorly controlled diabetes mellitus has been suggested as a contraindication for dental implant therapy2,3 because of its substantial effect on successful implant osseointegration.4 Diabetes mellitus has been closely associated with disorders in skeletal physiology and osseous healing,5 collectively referred to as “diabetic bone disease” or “diabetic osteopathy”6 and characterized by osteopenia,7,8 decreased bone mineral content,9,10 and delayed fracture healing.11 Osteopenia is likely to result in diminished bone formation, and studies have demonstrated diminished bone formation in experimental bone defects,12,13 as well as delayed bone regeneration in extraction sockets,14 inhibited or delayed peri-implant bone formation and osseous integration,15-22 and reduced peri-implant bone density23 in diabetic animals. Reduced removal torque values have also been observed postimplantation,24 showing the effect of experimental diabetes on the biomechanical properties of endosseous implants. The altered bone response found in diabetes has been associated with reduced osteoblastic activity25–31 related to diabetic osteopenia.28 The clinical ramifications of such findings may include an increased risk of implant failure in diabetic patients compared with healthy controls32,33. Moreover, trends of increased early vs late implant failure34 and an increased failure rate after functional loading35,36 have also been reported.

Advanced glycation end products (AGEs) cause many complications of diabetes,37,38 apparently as a result of the high levels that accumulate in living tissues. This phenomenon links numerous diabetic complications, through induction of marked changes in cellular and extracellular matrix components, and has also been documented in bone tissues.12,39 Administration of AGEs to calvaria defects in normal mice inhibits bone healing in vivo, and mimics inhibited bone formation in diabetic animals.12 The receptor for advanced glycation end products (RAGE) is present in osteoblasts, and elevated levels of RAGE have been observed in healing tissues in diabetic animals.12 AGE/RAGE interactions result in increased apoptosis of
mesenchymal cells, particularly primary rat calvaria and murine MC3T3-E1 osteoblasts, and have the potential to affect the growth and function of osteoblasts and to impair the organization and mineralization of extracellular matrix. Collagen is a major protein of the bone organic matrix and undergoes intra- and extracellular posttranslational modifications to form a functional extracellular matrix. Thus, lysyl oxidase–dependent collagen cross-linking is essential for bone strength. Elevated glycation of collagen, which occurs in diabetes, interferes with discoidin domain receptor-2 (DDR2) binding and activation, resulting in failure to maintain lysyl oxidase levels made by osteoblasts. DDR2 binding and activation are disrupted by collagen glycation, pointing to an alternative mechanism for the diminished levels of lysyl oxidase and consequently low lysyl oxidase–derived cross-links in diabetic bone. Taken together, these studies suggest that osteoblast differentiation may be inhibited in diabetes and that reduced osteoblast differentiation could be a major mechanism contributing to the observed reduced osteoblast function and reduced bone healing in diabetes.

If inhibited osteoblast differentiation is the principal mechanism of inhibited diabetic bone formation, it follows that application of factors known to stimulate osteoblast differentiation could potentially reverse and normalize diminished bone healing in diabetes. To test this idea, an experiment was undertaken in which a factor known to promote osteoblast differentiation, recombinant human bone morphogenetic protein 2 (rhBMP-2), was applied to calvaria defects in diabetic mice, and the effect on bone healing was evaluated. The goal of the current study was to determine whether the controlled local application of rhBMP-2 restores intramembranous bone healing to normal levels in diabetic animals.

**MATERIALS AND METHODS**

**Diabetes Induction and Characterization**

Experiments were performed in BALB/cByJ mice (Jackson Laboratories) as previously described in accordance with the guidelines of the U.S. National Institutes of Health for the care and use of animals for experimental procedures. Eight-week-old male animals were maintained according to approved protocols of Boston University. Animals were given free access to tap water and an NIH 31M mouse diet (SK52, Purina Mills). Generation of diabetic and control non-diabetic animals was accomplished using the multiple low-dose streptozotocin methodology. The number of animals per experimental group was eight, unless otherwise indicated.

The diabetic condition was characterized. Blood glucose (Accu-Check Advantage, Roche Diagnostics) and urine glucose levels (Multisix 10SG reagent strips, Bayer) were monitored twice weekly throughout the typical 33-day experimental period, and the onset of diabetes by experimental day 12 was confirmed in all diabetic animals utilized (blood glucose levels of at least 250 mg/dL). Blood and urine glucose values for control animals were normal (100 to 110 mg/dL). Protein and ketones in the urine were assayed twice weekly and were not detected (Multisix 10SG reagent strips, Bayer). Levels of glycated hemoglobin in blood (Glyc-Affin, Wallac) and levels of insulin in serum (Linco Research) were measured at sacrifice.

**Experimental Bone Defects**

Sixty-four animals were used. Diabetes was induced in half of the animals. Surgical procedures were performed 1 week after confirmation of diabetes (blood glucose > 250 mg/dL on experimental day 18); one 2.1-mm-diameter lesion was created in each parietal bone. BMP-2 or buffer alone was applied to acellular collagen sponges (ACS) (Genetics Institute), and 2-mm-diameter ACS disks containing the specified amount of rhBMP-2 were applied to the calvaria defects within 2 hours of preparation. Normal and diabetic animals were separated into four treatment modalities to be tested, resulting in a total of eight groups of animals: 1.8 μg of rhBMP-2 per defect (n = 8), 0.4 μg of rhBMP-2 per defect (n = 8), ACS loaded with buffer only (n = 8), and controls receiving no sponges (n = 8). Flaps were sutured, and postoperative care was performed. All the animals were sacrificed 14 days after the surgical procedure. The surgical areas were dissected free, fixed in formalin, and processed for histologic and histomorphometric analyses.

**Histologic and Histomorphometric Evaluation**

Tissues were sectioned perpendicular to the plane of calvarial bone. Histologic slides were stained with hematoxylin-eosin (H&E) for the evaluation of new bone and with Masson Trichrome for the evaluation of residual ACS carrier and nonmineralized connective tissues. The three most central sections of each defect were analyzed. The amounts and quality of regenerated bone tissue, cellularity, vascularity pattern, and degree of inflammation were evaluated. Linear measurements were done with an image analysis system (ImagePro 4.0). Bone ingrowth from the rims of the initial defects toward its center was quantified. Bone bridging was expressed as a percentage of the original defect width. Measurements included: (a) the distance between the rims of the initial bone defect, and (b) the distance between the rims of the remaining defect. The amount of bone bridging was calculated as: \( \frac{(a - b)}{(a) \times 100} \).
The areas of regenerated bone, cartilage, and residual ACS carrier present in the healed defects were measured from digitally captured images of stained slides with the ImagePro 4.0 software. The boundaries of the features of interest were traced with a mouse-driven cursor on video images on the display monitor with a handheld mouse. The area of the outlined image was then calculated electronically with the software. Bone area was measured on three slides for each bone defect from each animal. The readings were averaged to obtain the mean for every animal, which was used as the unit for statistical analyses. Results were presented as means ± standard deviations (SDs).

Statistics
The biochemical measurements were analyzed with one-way analysis of variance (ANOVA). Post hoc analysis was performed with the Student t test for ordinal variables and with the Wilcoxon signed rank test for cardinal variables. A value for $\alpha$ of 95% or higher was used to indicate statistical significance. The histomorphometric measurements for area determinations were analyzed with one-way ANOVA. Post hoc statistical testing was performed using the Bonferroni method, and an $\alpha$ of 95% or higher was used to indicate statistical significance.

RESULTS
All animals injected with streptozotocin became diabetic by day 11, and the characteristics of diabetes in this model are summarized in Table 1. The data show that the diabetic state was not accompanied by extreme metabolic dysregulation. After 14 days of healing, the calvarial defects demonstrated differences in healing as a function of diabetes. Bone bridging (Table 2) was inhibited by approximately 50% in diabetic animals compared to nondiabetic controls (Table 2, groups 1 and 2; $P = .004$). The application of ACS with adsorbed rhBMP-2 to bone defects in diabetic animals stimulated bone bridging and bone regeneration and was dose-dependent (Table 2). ACS without rhBMP-2 did not stimulate but instead inhibited healing somewhat, demonstrating that stimulation of healing depends directly on rhBMP-2. Representative micrographs of lesions treated with the collagen carrier with or without BMP-2 are shown in Figs 1a to 1f. Both doses of rhBMP-2 were effective in stimulating diabetic bone healing, but there was a tendency toward increased bone bridging in defects that received the higher dose of rhBMP-2 (Table 2, groups 3 and 4; $P = .05$).

Nondiabetic animals exhibited a more robust response to rhBMP-2, as expected. For example, the defects in diabetic animals implanted with the lower dose of rhBMP-2 exhibited a tendency toward less bridging compared to nondiabetic animals implanted with either dose, further illustrating the inhibitory effect of type 1 diabetes on bone formation (Table 2, groups 3 and 5; $P = .05$). Application of rhBMP-2 significantly stimulated bone regeneration in nondiabetic animals compared to untreated nondiabetic controls with and without ACS applied to the defects (Table 2, groups 5, 6, and 8; $P < .001$). All the defects in nondiabetic animals that were grafted with rhBMP-2 were completely bridged, irrespective of the dose applied (Table 2, groups 5 and 6; $P > .05$). Bone bridging in the defects in diabetic animals treated with the higher dose of rhBMP-2 was also complete and showed no significant difference versus the bridging observed in normal...
animals treated with either dose (Table 2, groups 4 to 6). Differences in bridging in defects in nondiabetic and diabetic animals implanted with the ACS without rhBMP-2 were not significant (Table 2, groups 7 and 8; \( P = .122 \)); however, bridging in these groups was significantly less than in untreated nondiabetic animals \( (P = .001) \). This indicates that the unloaded ACS actually inhibited healing slightly in all animals. Taken together, these data support the hypothesis that calvarial defect healing is inhibited in diabetes, that rhBMP-2 stimulates bone bridging in diabetic animals in a dose-dependent manner, and that the resulting bone bridging is complete and approaches the degree of bridging that occurs in normal animals treated with rhBMP-2.

Bone bridging is a linear measurement and does not fully reflect the degree of accumulated healing bone. Thus, area measurements of new bone were performed to analyze more closely the ability of rhBMP-2 to stimulate bone healing in diabetes. The exogenous application of rhBMP-2 significantly enhanced the area of regenerated bone in diabetic animals. Defects in diabetic animals that received either dose of rhBMP-2 exhibited significantly more bone regeneration than untreated defects and defects implanted with the ACS carrier (Table 2). Defects in nondiabetic animals implanted with 1.8 \( \mu \)g of rhBMP-2 exhibited significantly more regenerated bone area than defects in diabetic animals implanted with either rhBMP-2 dose (Table 2). Defects in nondiabetic animals implanted with 0.4 \( \mu \)g of rhBMP-2 exhibited significantly more bone regeneration than diabetic animals treated with the same rhBMP-2 dose (Table 2). No significant difference was found in the area of regenerated bone in ACS-treated defects of normal and diabetic animals (Table 2, groups 7 and 8; \( P = .7 \)). Taken together, these results suggest that rhBMP-2 stimulated bone healing in diabetic animals during a 14-day healing period, but the amount of new bone formed by area measurements was less than what formed in normally healing lesions stimulated with rhBMP-2.

Defects that received the rhBMP-2 implants exhibited some degree of residual ACS carrier populated by nonmineralized connective tissue, irrespective of the dose implanted, in both normal and diabetic animals. Bone defects in normal animals implanted with rhBMP-2 exhibited significantly more bone regeneration than untreated defects and defects implanted with the ACS carrier (Table 2). Defects in nondiabetic animals implanted with 1.8 \( \mu \)g of rhBMP-2 exhibited significantly more regenerated bone area than defects in diabetic animals implanted with either rhBMP-2 dose (Table 2). Defects in nondiabetic animals implanted with 0.4 \( \mu \)g of rhBMP-2 exhibited significantly more bone regeneration than diabetic animals treated with the same rhBMP-2 dose (Table 2). No significant difference was found in the area of regenerated bone in ACS-treated defects of normal and diabetic animals (Table 2, groups 7 and 8; \( P = .7 \)). Taken together, these results suggest that rhBMP-2 stimulated bone healing in diabetic animals during a 14-day healing period, but the amount of new bone formed by area measurements was less than what formed in normally healing lesions stimulated with rhBMP-2.

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Defects that received the rhBMP-2 implants exhibited some degree of residual ACS carrier populated by nonmineralized connective tissue, irrespective of the dose implanted, in both normal and diabetic animals. Table 3 shows the results of quantitative histomorphometric analyses of the area of residual soft tissues within the defects treated with rhBMP-2. As shown, diabetic animals implanted with rhBMP-2 exhibited significantly decreased bone density versus normal animals as a result of inhibited osteogenesis and increased soft tissue area in the regenerated bone. The higher dose of rhBMP-2 elicited a more robust osteogenic response and a lesser degree of soft tissue formation (Table 3 and Figure 2), partially compensating for the effects of diabetes in bone formation.
DISCUSSION

Among the well-known metabolic consequences of diabetes, concerns have been raised regarding its deleterious effects on bone and mineral metabolism. This study demonstrates that rhBMP-2 can partially correct the inhibited bone healing caused by type 1 diabetes. The nature of bone healing in diabetic animals was qualitatively different from that found in nondiabetic animals. Standardized bone defects in nondiabetic animals regenerated predominantly new bone, whereas bone defects in diabetic animals regenerated a combination of bone, residual ACS, and connective tissue.

Experimental studies have demonstrated a significant negative impact of diabetes in alveolar bone biology⁴⁹–⁵¹, inhibited formation of the collagenous

Table 3  Histomorphometric Analyses of Tissue Areas (± SDs) in Calvarial Defects in Diabetic and Nondiabetic Animals Treated with rhBMP-2

<table>
<thead>
<tr>
<th>Group</th>
<th>Soft tissue (mm²)</th>
<th>Bone area (mm²)</th>
<th>Bone density (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Diabetic (+ ACS)</td>
<td>0.74 ± 0.09</td>
<td>0.14 ± 0.009</td>
<td>15.91 ± 3.21</td>
</tr>
<tr>
<td>2. Normal (+ ACS)</td>
<td>0.62 ± 0.13</td>
<td>0.19 ± 0.01</td>
<td>24.46 ± 8.21</td>
</tr>
<tr>
<td>3. Diabetic + 0.4 µg BMP-2</td>
<td>0.61 ± 0.21</td>
<td>0.47 ± 0.19</td>
<td>43.52 ± 16.73</td>
</tr>
<tr>
<td>4. Diabetic + 1.8 µg BMP-2</td>
<td>0.54 ± 0.12</td>
<td>0.75 ± 0.17</td>
<td>58.14 ± 13.26</td>
</tr>
<tr>
<td>5. Normal + 0.4 µg BMP-2</td>
<td>0.44 ± 0.13</td>
<td>0.66 ± 0.19</td>
<td>60.00 ± 26.31</td>
</tr>
<tr>
<td>6. Normal + 1.8 µg BMP-2</td>
<td>0.27 ± 0.08</td>
<td>1.01 ± 0.25</td>
<td>78.91 ± 14.17</td>
</tr>
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Figs 2a to 2f  Higher-magnification views of the center of the defects treated with ACS carrier with or without rhBMP-2 (original magnification ×400).

Fig 2a  Nondiabetic animal, ACS carrier (H&E).

Fig 2b  Diabetic animal, ACS carrier (H&E).

Fig 2c  Nondiabetic animal, ACS carrier loaded with 0.4 µg rhBMP-2 (Masson trichrome).

Fig 2d  Diabetic animal, ACS loaded with 0.4 µg rhBMP-2 (Masson trichrome).

Fig 2e  Nondiabetic animal, ACS loaded with 1.8 µg rhBMP-2 (Masson trichrome).

Fig 2f  Diabetic animal, ACS loaded with 1.8 µg rhBMP-2 (H&E).
framework in extraction sockets, delayed alveolar healing,51 reduced bone formation and turnover in the alveolar wall surrounding the root,50 reductions of osteocyte density, and an increase in empty lacunar density have been observed in the alveolar bone of diabetic animals.49 Most of these changes are attributed to the osteopenic state of diabetic bone. Osteopenia is a complication of type 1 diabetes7 that results in reduced osteoblastic activity25–30 and decreased bone mineral content9,10 and is likely to result in diminished bone formation. Studies have demonstrated diminished bone formation in experimental bone defects,1–12 as well as delayed bone regeneration in extraction sockets,14 inhibited osseous integration of implants,18 and higher variability of outcomes and an increased rate of infectious complications following guided bone regeneration procedures52 in type 1 diabetic animals, thus illustrating the possible negative effects of diabetes on bone healing related to implant therapy. Interestingly, it has been demonstrated that alterations in the extracellular presence of proinflammatory cytokines and growth factors within diabetic tissues may delay the proliferation and differentiation of mesenchymal progenitor cells and osteoblasts in the osseointegration process.55 It has been suggested that continuous application of growth factors, such as fibroblast growth factor-2, may facilitate osseointegration and bone healing in conditions of locally and systemically inhibited osteogenesis such as that found in diabetes.1 Thus, targeted controlled local delivery of biologically active substances may stimulate bone regeneration and compensate for the inhibitory effects of diabetes on bone healing. Biologically enhanced therapies may offer significant clinical benefits by enhancing the endogenous healing capacity of bone defects, increasing the rate and total amount of bone formation and ultimately resulting in significantly improved bone regeneration, especially in situations of locally or systemically inhibited healing, such as that observed in diabetes mellitus.

The role of BMP-2 in normal tissue repair has been investigated, and it has been demonstrated that BMP-2 is a critical endogenous mediator of the signaling cascade that governs bone repair.54 Previous studies have proposed that suppressed osteoblastogenesis may be a cause of the reduced expression of the osteoblast-specific genes, such as BMP-2, and thus a mechanism for low bone mass, lower mineralized bone volume/tissue volume, and impaired bone regeneration in rodent models of diabetes mellitus.55–57 Despite the fact that the anabolic effect of rhBMP-2 in standardized bone defects in experimental normal animal models has been well documented,58–60 to the authors’ knowledge, no studies have yet investigated the degree to which rhBMP-2 can mitigate against the effects of diabetic osteopenia or whether rhBMP-2 can reverse inhibited bone formation in diabetes. The current authors reasoned that rhBMP-2 would be highly effective in reversing the effects of diabetes on bone formation because it is a stimulator of osteoblast differentiation, rather than a direct stimulator of osteoblast function and extracellular matrix production.47,64

The current results show that rhBMP-2 was quite effective in reversing the diabetes-induced inhibition of bone healing, but the new bone that formed was qualitatively different from that found in nondiabetic animals in two respects. First, the area of bone formed in diabetic animals was less than normal; second, a greater amount of nonmineralized tissues was found in healing diabetic lesions, resulting in diminished bone density in diabetic animals. These results demonstrate that bone regeneration modulated by rhBMP-2 in diabetic animals may replicate and up-regulate the biologic events of bone metabolism and repair in these animals.55

A previous study12 proposed a role for AGE/RAGE interactions as a possible mechanism for impaired bone healing in vivo. It is of interest that AGE/RAGE interactions cause increased apoptosis of mesenchymal cells, notably fibroblasts,40 primary rat calvaria, and murine MC3T3-E1 osteoblasts.41 AGEs accumulated in the bone matrix have the potential to affect the growth and function of osteoblasts and impaired matrix mineralization39 by activation of the AGE/RAGE pathway39 and to suppress the osteogenic properties of osteoblasts in vivo.42 The decreased osteoblastogenesis seen in diabetes may be associated with the regulation of intracellular signaling molecules, resulting in decreased expression of genes relevant to osteoblastic phenotypic development.43,44 Knowledge of the multiple mechanisms by which AGE/RAGE interactions alter osteoblast differentiation, survival, and function seems likely to permit development of novel multifaceted therapeutic strategies to address the osteopenia that is seen as a complication of type 1 diabetes.

Taken together, the findings of the present study suggest some important clinical considerations. Uncontrolled diabetes results in significantly reduced regenerative bone formation, both untreated and assisted by application of rhBMP-2, which is manifested as inhibited osseointegration caused by reduced area and calcification of formed bone, as well as reduced contact between bone and implant.16,17,20,21 Targeted controlled local delivery of biologically active substances such as rhBMP-2 may stimulate bone regeneration and compensate for the inhibitory effects of diabetes on bone healing and osseointegration. Biologically enhanced procedures may offer significant clinical benefits by enhancing the endogenous healing capacity of bone defects, thereby increasing the rate and total amount of bone formation and ultimately resulting in significantly improved bone regeneration.
CONCLUSION

This study demonstrates that recombinant human bone morphogenetic protein 2 (rhBMP-2) significantly stimulates bone regeneration in vivo and partially restored the impaired bone healing in diabetic animals to levels similar to normal untreated animals. These properties of rhBMP-2 bone defect therapy may have potential clinical use for stimulation of bone regeneration in normal as well as diabetic patients. Qualitative differences in the healing potential of bone defects in normal and diabetic animals likely reflect a condition of multifactorial etiology that deserves additional investigation focused on mechanistic aspects of these phenomena. Local delivery of rhBMP-2 in combination with other therapeutic factors that address additional mechanistic features of diabetic osteopenia may be developed as novel therapeutic approaches to address impaired bone healing in diabetic patients in need of bone reconstruction.

ACKNOWLEDGMENT

This study was supported by NIH/NIDCR grant R01 DE011004 (Phillip C. Trackman). The authors reported no conflicts of interest related to this study.

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The International Journal of Oral & Maxillofacial Implants 713

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