Effects of local delivery of bFGF from PLGA microspheres on osseointegration around implants in diabetic rats

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Objective. Diabetes mellitus may impair bone healing after dental implant placement. The objective of this study was to evaluate the effects of the local delivery of basic fibroblast growth factor (bFGF) from poly(lactide-co-glycolide) (PLGA) microspheres on osseointegration around titanium implants in diabetic rats.

Study Design. The bFGF-PLGA microspheres were prepared by the W/O/W double-emulsion solvent evaporation method. A total of 20 rats were used to create diabetic animal models by giving them a high-fat and high-sugar diet and a low-dose streptozotocin intraperitoneal injection. Titanium implants were planted into the tibias of the diabetic rats and into 10 normal rats. Microspheres were loaded on the surfaces of the implants in the bFGF intervention group before they were placed into the rats. After 4 or 8 weeks, the tibias containing the implants were removed and embedded with resin. Uncalcified tissue slices were prepared to compare osseointegration.

Results. At 4 weeks, the bone-implant contact rate in the diabetic control group was less than that in the control group and the bFGF intervention group ($P < .05$). At 8 weeks, the results among the 3 groups were similar to those at 4 weeks.

Conclusions. The local delivery of bFGF from PLGA microspheres into areas around titanium implants may improve osseointegration in diabetic rats. (Oral Surg Oral Med Oral Pathol Oral Radiol 2012;114:284-289)

Dental implants have become an ideal treatment modality in patients with missing teeth. However, in patients with diabetes mellitus, such implants are associated with a high failure rate.[1] Studies have shown that implant success rates in diabetic patients range from 85.6% to 94.3%, which is lower than the rates in nondiabetic patients.[2] Diabetes can affect the bone, eventually resulting in osteopenia and impaired healing of fractures.[3] Osteopenia is thought to be a contributing factor to the increased risk of fractures in diabetic patients and leads to the increased resorption of bone.[4] As such, implants may be contraindicated in these patients.[5,6]

Growth factors are polypeptides that can either stimulate or inhibit cellular proliferation, differentiation, migration, adhesion, and gene expression. Recent in vitro and in vivo studies have demonstrated the osteoinductive effects of different growth factors.[7] Basic fibroblast growth factor (bFGF) plays an important role in morphologic and bone-healing processes and is a potent stimulator of osteoblastic proliferation.[8,9] Studies have confirmed that bFGF regulates extracellular matrix production by osteoblastic cells in vitro; bFGF that was systemically administered in vivo resulted in increased endosteal bone formation in rats.[10] Chen et al. found that the local application of FGF-2 in fractured bones had significant positive effects on bone healing.[11] Santana et al. reported that the loading of FGF-2 in the cranial fossa surrounding implants in diabetic rats showed better osseointegration than the control group, with new bone formation significantly increased.[12] In addition, the large clinical VIVA (Vascular Endothelial Growth Factor in Ischemia for Vascular Angiogenesis) trials using bFGF have proven its safely and efficacy.[13,14] However, because the half-life of bFGF is very short, its potential application may have limited results in terms of continued therapeutic efficacy.[15]

Poly(lactide-co-glycolide) (PLGA), which is a biodegradable material owing to its good biocompatibility, has been used as a carrier of microspheres to parcel drugs and to lengthen the sustained release time.[16] As such, PLGA has been widely used in the pharmaceutical industry.[17] In earlier studies, the bFGF-PLGA microsphere drug delivery system has been demonstrated to contribute to successful osteoinduction in fractures involving animal models.[18,19]

In the present study, we used PLGA-packaged bFGF microspheres loaded on the surfaces of implants, which were inserted into the tibias of diabetic rats, to evaluate whether the controlled local application of bFGF re-

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stored the tibias of diabetic rats with placements of dental implants.

MATERIALS AND METHODS

Preparation of the bFGF delivery system

The bFGF (PeproTech)–loaded PLGA microspheres were prepared using the double-emulsion solvent evaporation method (W/O/W). This method involved 10 μg lyophilized bFGF being dissolved into 0.1 mL distilled water (internal aqueous phase, W1). Subsequently, PLGA (L/G ratio 50:50; DaiGang, Ji'nan, China; 500 mg) and 0.14 g Span 80 (sorbitan oleate; Chemical Industry, Beijing, China) were dissolved in 10 mL dichloromethane (oil phase, O). Later, W1 was dissolved in (O) and emulsified using a high-speed homogenizer for 60 seconds to form the W/O emulsion. This primary emulsion was reemulsified with the external aqueous phase (reemulsification solution, W2), which contained 40 mL 2% polyvinyl alcohol aqueous (PVA) solution, using a high-speed digital homogenizer at 12,000 rpm for 5 minutes. The obtained W1/O/W2 emulsion was stirred at 200 rpm for 8 hours with a propeller stirrer to volatilize the surplus solvent and to harden the microspheres. Subsequently, the obtained solid microspheres were washed and collected by filtration and centrifugation. Finally, the microspheres were freeze-dried and stored at −20°C in desiccative condition.

The morphology of the bFGF-loaded PLGA microspheres was examined by scanning electron microscopy. The release kinetics of bFGF in vitro was determined. To correspond with the internal environment of the human body, bFGF microspheres (50 mg) were incubated with constant shaking at 100 rpm in 10 mL phosphate-buffered saline solution (PBS) at 37°C for 28 days in a closed borosilicate glass tube. The solutions were then collected and changed on days 1, 2, 3, 5, 7, 10, 14, 17, 21, 24, and 28. A sample of 3 mL was taken each day, and an equal amount of fresh PBS was added and incubated. Adopting the enzyme-linked immunosorbent assay determination absorbance of the (*A*) value of the bFGF standard substance and sample solution, the bFGF concentration and (*A*) value were proportional to the standard curve. Using the standard curve, the release quantity of the bFGF microsphere was calculated; using the accumulated release rate, the microsphere's release kinetic curve was also obtained.

Animals

A total of 35 9-week-old male specific pathogen–free (SPF)–level Sprague-Dawley rats, with an average weight of 240 g, were purchased from Laboratory Animal Resources of The Fourth Military Medical University (Xi'An, China). The animal experiments were approved by the Animal Welfare Committee of the Fourth Military Medical University in strict accordance with the policies and principles of the Animal Welfare Act and according to the recommendations set forth by the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, Washington, DC). Ten of the total 35 rats were randomly selected as the nondiabetic control group after adapting their feeding for 1 week, whereas the others were established as the diabetes molding group.

The diabetes molding rats were fed with a diet enriched with fat and glucose, of which the detailed formula included basic feed (66.5%), sucrose (20%), lard (10%), cholesterol (2.5%), and sodium cholate (1%). After 4 weeks of high-energy feeding, diabetes was induced in the rats by intraperitoneal injections of streptozotocin (STZ) at 30 mg/kg body weight in distilled water. The weights and blood glucose levels of all rats were monitored at least once a week. Blood was drawn from the tail veins, and glucose was measured using a glucometer (Beijing Yicheng, China). Glucose concentrations >16.7 mmol/L were defined as a successful induction of diabetes. The hyperglycemic rats were continued on diets that were high in fats and sugars. We rejected rats that experienced failed molding and death and eventually obtained 20 diabetic rats whose blood glucose levels ranged from 17.0 to 20.5 mmol/L. The diabetic rats were then randomly divided into 2 groups of 10 each: the diabetes control group and the bFGF experimental group.

Implantation

The animals were anesthetized by intraperitoneal injections, at 0.25 mL/100 g body weight, of 2% pentobarbital sodium. The right legs were shaved and sterilized with 1% iodine. Then, a 2-cm full-thickness incision was made in the right tibia to expose the anterior-medial portion. A hole was prepared 3 mm below the knee joint into the tibial bone using 2.2-mm, 2.8-mm, and 3.0-mm drills activated by a surgical micromotor (1,000 rpm) and irrigated with 0.9% sterile saline solution. Then a sterilized titanium root-form implant (3.3 mm × 6 mm) with a microarc oxidation–treated surface was press-fitted into position; its stability was confirmed by passive mechanical retention. Before inserting the implant in the bFGF experimental group, we effectively distributed the previously prepared pulverized microspheres in 0.5 mL of blood and soaked the implant in the mixture until a clot was formed on the surface of the implant (Fig. 1). The microspheres were immediately released directly into the bone around the implants. The incision was closed with a conventional suture. Postoperative antibiotic treatment with 6 mg gentamycin/kg body weight was injected twice daily for 3 days.

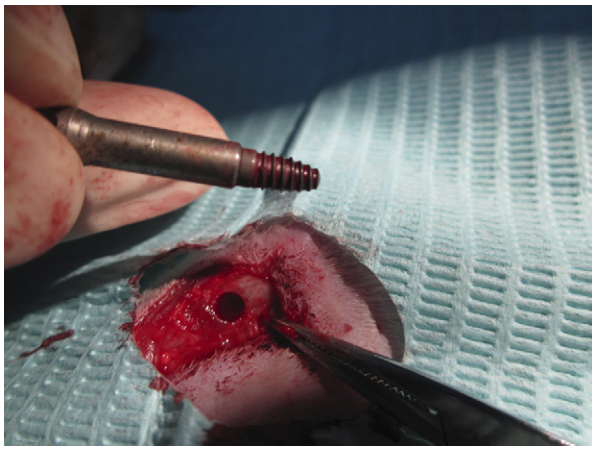

Fig. 1. The implant being placed in the rat tibia.

Histologic specimens

At 4 or 8 weeks after the implantation, the animals were killed with an overdose of anesthetic solution, and the tibias containing the implants were dissected from the soft tissue and fixed in 4% buffered formalin. After dehydration with ethanol, the samples were embedded in resin, sectioned longitudinally using a heavy-duty microtome (Leica SP1600; Hindenburg, Germany), and stained with Masson Trichrome. Later, a biologic fluorescence microscope (Leica DM 6000B) was used to collect images, which were analyzed with the Leica Imaging System. The bone-implant contact rate (BIC) was used as the index of osseointegration, equal to the surface length contact of the implant with the bone/the overall surface length of implant inserted into the bone.

Statistical analysis

Taking the BIC for each side of the implants as the source data, a 1-way analysis of variance was used to assess the differences between the groups with the help of the Statistical Package for the Social Sciences (SPSS version 13.0 for Windows; Chicago, IL). Pairwise comparisons between the groups were obtained using the Student-Newman-Keuls test. Probabilities of $<.05$ were accepted as significant.

RESULTS

bFGF-PLGA microsphere morphology and in vitro release kinetics

A scanning electron microscope image of the bFGF microspheres is shown in Fig. 2. The morphology of the surfaces of the microspheres was uniform and sleek. The average diameter of the microspheres was 1.138 ± 0.304 μm, as measured by a fiber-optic particle analyzer. As shown in Fig. 3, the early release kinetics of the microspheres was fast, with release rates of 25.2% and 45.3% on days 1 and 3, respectively. Approximately 70% of the bFGF was released from the PLGA microspheres during the first 14 days; after 28 days, the cumulative release rate reached 82%.

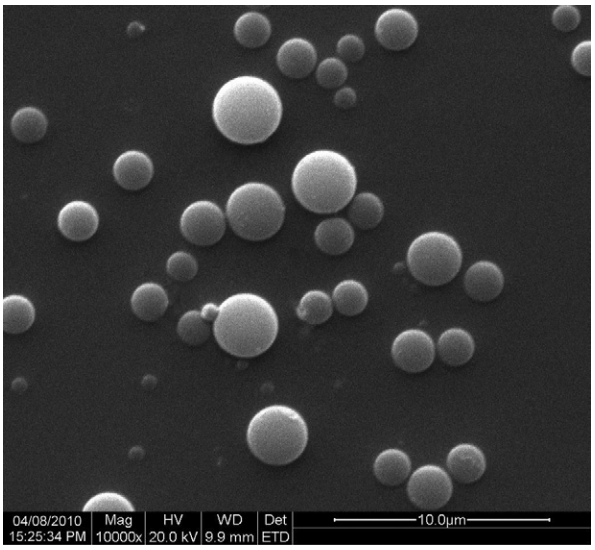

Fig. 2. Scanning electron microscope image of basic fibroblast growth factor 2–poly(lactide-co-glycolide) microspheres (×10,000 magnification).

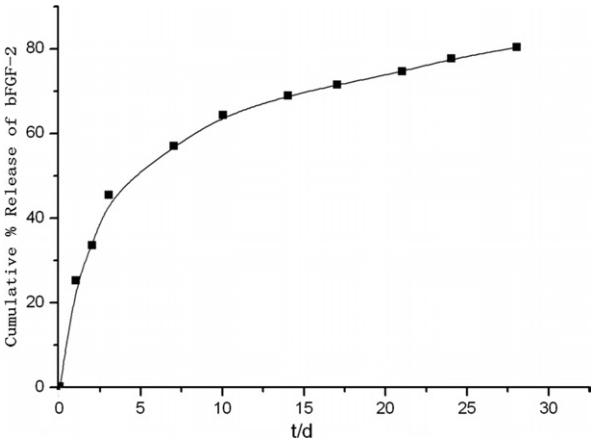

Fig. 3. In vitro release profile of basic fibroblast growth factor 2–PLGA microspheres.

General conditions of the animals

After a week of intraperitoneal STZ injection, the blood glucose levels in the diabetic rats were significantly higher than those of the control group ($P < .05$). Their urine outputs had significantly increased, and the bedding had to be replaced every day. The 20 successful rat diabetes models presented with yellow pelage and a lack of luster. Until they were killed, all of the animals were able to move freely. In addition, no rats suffered from infections or other diseases. Throughout the course of the experiment, the blood glucose levels of

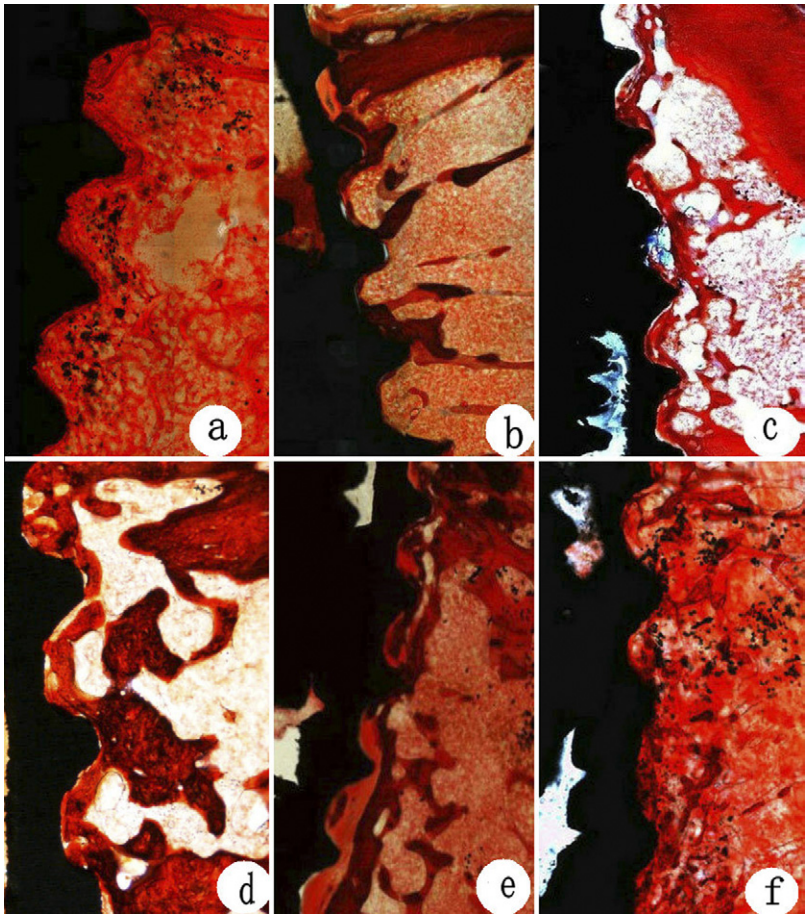

Fig. 4. Histologic observation of each group at 4 and 8 weeks (Masson Trichrome, ×40 magnification). **a,** Normal control group at 4 weeks; **b,** diabetes control group at 4 weeks; **c,** basic fibroblast growth factor 2 (bFGF-2) experimental group at 4 weeks; **d,** normal control group at 8 weeks; **e,** diabetic control group at 8 weeks; **f,** bFGF-2 experimental group at 8 weeks.

the control group rats were always <8.5 mmol/L, whereas the diabetic rats' blood sugar levels were maintained at a range between 16.9 and 22.5 mmol/L.

Histomorphologic observations

Four weeks after implantation, the longitudinal tissue slices with the titanium implants showed that a newly formed thin bony layer had surrounded the implants in the normal control group (Fig. 4, *a*) and in the bFGF experimental group (Fig. 4, *c*), although less was seen in the diabetic control group, in which the bone was loosely organized and a woven area was seen (Fig. 4, *b*). At week 8 after surgery, each group revealed trends similar to those at week 4 after implantation, although there was still less bone-implant contact in the diabetic control group (Fig. 4, *e*). The implants in the control group (Fig. 4, *d*) and the bFGF intervention group (Fig. 4, *f*) were osseointegrated, which appeared to involve more mature bone and exhibited more extensive bone formation.

Histomorphometric outcome

The results of the histomorphometric measures are shown in Fig. 5. At 4 weeks, the BIC was 44.8 ± 4.3% in the diabetic control group, which was significantly ($P < .05$) lower than the values of 57.9 ± 6.7% observed in the normal control group and 53.2 ± 5.6% in the bFGF intervention group. At 8 weeks, the BICs of all of the groups had increased, with the average BIC in the normal control and bFGF intervention groups reaching 68.2 ± 5.2% and 63.7 ± 4.4%, respectively. Both levels were significantly ($P < .05$) greater than the value of 50.4 ± 5.1% in the diabetic control group.

DISCUSSION

With the advent of modern lifestyles, the incidence of diabetes has increased sharply, and nearly 90% of cases involve type 2 diabetes mellitus (e.g., non–insulin-dependent diabetes mellitus).[20] Compared with the general population, patients with diabetes are confronted with an increased risk of dental implant failures.[1,2,5,6,21] Achiev-

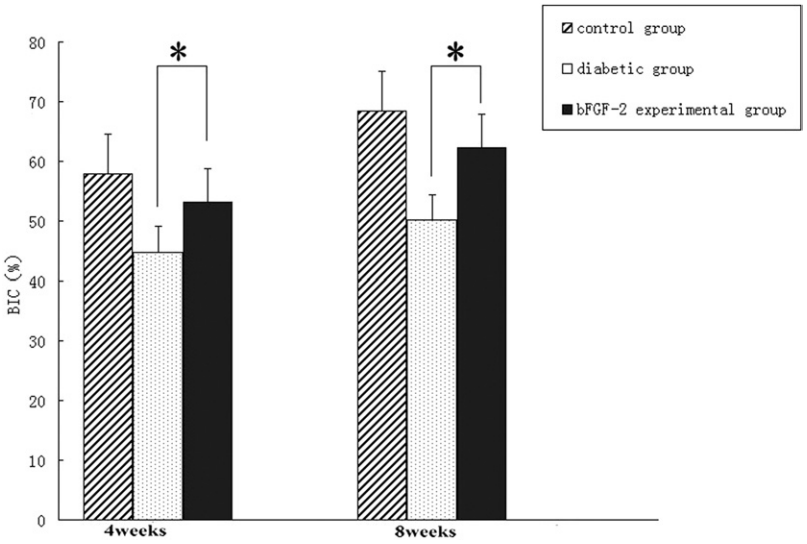

Fig. 5. Bone-implant contact rate (BIC) of each group. *$P < .05$; compared with control group.

ing success with implant prostheses in diabetic patients who have lost teeth has a very important clinical significance. The main influential factors of osseointegration in the diabetic population include delayed bone healing, bloodstream obstruction, decreased immune response, decreased formation of collagen fibers, and strengthened activity of collagenase.[1,22,23] The results produced by these factors include poor osteoblastic differentiation and limited new bone formation.

In the present study, we used the methods of Abdin et al., which involve feeding SD rats with a diet high in fat and sugar for 4 weeks and administering intraperitoneal injections of small doses of STZ to create type 2 diabetic rat models.[24] The high–fat and sugar diet resulted in the rats' becoming insulin resistant. The subsequent intraperitoneal injections of small doses of STZ led to damage to part of the functioning beta cells, resulting in peripheral organization insensitivity to insulin. The combination of these 2 procedures induced diabetes in the rats with changes similar to those seen in human type 2 diabetes, both pathologically and physiologically, including hyperglycemia, hyperinsulinemia, reduced glucose tolerance, and insulin resistance.

In earlier experience with an FGF controlled-release system, Gomez et al. using poly-L/D-lactide (PLDLA) 96/4 scaffolds and FGF-1 to evaluate the healing of experimental bone defects.[25] In the present experiment, we manufactured bFGF-PLGA microspheres using the W/O/W method, and we controlled the temperature and the rotary speed, which may have strictly affected the microsphere performance conditions. The release kinetics of the microspheres showed that after 28 days, a small amount of bFGF was still being released from the microspheres in vitro, which achieved a well controlled release effect.

In earlier studies of dental implants in diabetic patients, some researchers applied drugs that could accelerate wound healing around implants to improve osseointegration (i.e., drugs containing insulin, protease inhibitors, plasma cellulose, metformin, etc.) in addition to a basic treatment strategy, such as the use of antibiotics and agents to control the blood sugar level.[22,23,26] In recent years, research involving drug delivery control release systems between the implant and bone has been a popular topic of discussion. Gao et al. combined zoledronic acid with bFGF loading on the implant surfaces of tibia bones of rats with osteoporosis and achieved satisfactory results.[27] Wang et al. used PLGA-packaged insulin-like growth factor 1 and the local delivery of insulin into the areas surrounding the implant in the tibias of spontaneous type 2 diabetes model Goto-Kakizaki rats.[28,29] Those authors also achieved ideal BICs. New bone formation around the implants was seen to mainly rely on blood clot organization, which guided the bone marrow cells that form the organic conjunction between the bone and the implants and allowed them to further differentiate into osteoblasts to achieve biologic ossification.[30]

In the present study, we mixed bFGF-PLGA microspheres with blood-forming clots and later coated them on the rough surfaces of implants, resulting in the release of bFGF from the blood clots. Therefore, bFGF influenced the bone around the implants via its biologic chemotactic action, creating bone marrow mesenchymal cells, macrophages, and fibroblasts that migrated toward the wound area and started the bone healing process. As a consequence, osteoblastic differentiation and collagen formation were promoted, which stimulated bone healing and improved osseointegration in the areas surrounding the implants.

CONCLUSION

The results of the present study show that the local delivery of PLGA-packaged bFGF microspheres around the implants may enhance early osseointegration in diabetic rats. Further studies are needed to show whether these properties of the bFGF PLGA microsphere control release system have the potential for clinical use to stimulate bone regeneration in human patients with diabetes.

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