Enhanced Bone-to-Implant Contact by Platelet-Released Growth Factors in Mandibular Cortical Bone: A Histomorphometric Study in Minipigs

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Purpose: *To determine the effects of platelet-released growth factors (PRGF) on bone-to-implant contact (BIC) in minipig cortical bone.* ***Materials and Methods:*** *In each of 8 adult minipigs, 2 implants were placed in the facial wall of the mandible, one implant with and one without PRGF. PRGF was defined as the supernatant from washed, thrombin-activated, allogenic, platelet-rich plasma cells obtained after centrifugation. Four animals were sacrificed at 4 weeks, and the remaining 4 were sacrificed at 8 weeks. For histomorphometric analyses, undecalcified ground specimens were prepared and stained with the Levai-Laczko stain.* ***Results:*** *For the entire follow-up time, 55.30% BIC was found with PRGF versus 38.91% without PRGF (P = .0198). At 4 weeks, BIC was 44.20% with PRGF versus 29.62% without PRGF (P = .0632), and at 8 weeks there was 70.36% BIC with PRGF versus 48.20% without PRGF (P = .1221).* ***Discussion:*** *Growth factors and other molecules released upon activation of platelet-rich plasma cells can enhance implant anchorage in cortical bone. PRGF obtained from allogenic sources does not impair healing.* ***Conclusion:*** *The results indicated that a single application of PRGF before implant placement can be sufficient to increase the percent BIC in minipig cortical bone.*
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Key words: *cortical bone, dental implants, platelet-derived growth factors, thrombin*

In past decades edentulous and partially dentate patients have often been rehabilitated with implant-supported fixed or removable prostheses. Fundamental studies conducted by Brånemark and coworkers showed that implant anchorage with direct bone contact can be achieved. This direct bone-to-implant interface was termed *osseointegration*.[1,2]

The success rate of osseointegration has been reported to be 81% in the maxilla and 91% in the mandible.[3] A detailed analysis of implant success rates has shown implant failures to correlate with bone quality.[4] Implant failure rates were found to be high in Class I mandibular bone, which consists mainly of cortical bone.[5] In cortical bone, resorption zones of host bone are larger and new bone forms slower than in cancellous bone.[6,7] Moreover, bone-implant contact (BIC) in cortical bone appears to decrease in the early healing phase.[8]

In cancellous bone, implant anchorage can be improved by the addition of thrombin-activated platelet-rich plasma (PRP).[9,10] Platelet-derived growth factor (PDGF), transforming growth factor-β, and vascular endothelial growth factor are stored in the alpha-granules of platelets and are released upon platelet activation.[11–14] In vitro studies have

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suggested that platelet-released growth factors (PRGF) alone may contribute to bone regeneration, as they are mitogenic for bone cells and periosteum-derived cells, induce osteoclast-like cell formation, and initiate angiogenesis.[12,14–17] Platelet-released growth factors can be prepared from PRP cells. Cells are washed and resuspended in an isotonic solution, and release of the platelet growth factors is initiated by the addition of thrombin and calcium.[14] After centrifugation, cell membranes remain in the pellet; the growth factors in the supernatant are termed PRGF.[18] Platelet-released growth factors has been prepared from 2×10^9 platelets/mL. This is 2 to 4 times more than the mean platelet count in PRP obtained by plasmapheresis.[19]

To the authors' knowledge, the effect of highly concentrated growth factors released from activated platelets on osseointegration has yet to be reported. Moreover, PRGF has not been tested in cortical bone, which is known to have poor regenerative potential.[6–8] It was hypothesized that highly concentrated PRGF can increase the BIC in cortical bone during the early phase of osseointegration. To test this hypothesis, implants were placed with and without PRGF in the facial wall of the minipig mandible and BIC was examined 4 and 8 weeks later by histomorphometric analysis.

MATERIALS AND METHODS

Preparation of PRGF

Platelet concentrates from 2 minipigs were prepared by standard apheresis using a triple blood bag system (Teruflec; Terumo Europe, Leuven, Belgium) filled with 63 mL citrated phosphate dextrose for stabilization. Approximately 450 mL of blood was withdrawn and the whole blood was centrifuged at 2,890 G for 6 minutes (Cryofuge 6000; Heraeus Sepatech, Osterode, Germany). Then the plasma was transferred manually to the second bag and the content was again centrifuged at 153 G for 12 minutes. The redundant platelet-poor plasma was transferred to the third bag and discarded. What remained in the second bag was about 30 ± 5 mL of platelet concentrate.[20] The platelet concentrate was washed in Tyrode's buffer, pH 6.4, and centrifuged at 1,400 G for 10 minutes. Pellets were resuspended in DMEM/F12 medium (Life Technologies, Grand Island, NY) resulting in platelet counts of 2×10^9/mL. Release of platelet factors into the supernatant was induced by adding 2 U/mL of human thrombin (Tissucol; Baxter, Vienna, Austria) for 30 minutes at room temperature. After centrifugation at 1,400 G for 10 minutes, the supernatant called PRGF was stored at –40°C.[18]

To determine whether PRGF retained its activity when stored frozen, a ^{3}[H]-thymidine incorporation was assayed in gingival fibroblasts. In these experiments, which have been described elsewhere,[20] aliquots of freshly prepared PRGF were frozen in liquid nitrogen for 10 minutes and assayed immediately after thawing.

Animals

Eight adult minipigs (4 males and 4 females), bred from Minnesota pigs and Vietnamese potbellied pigs, were used for the experiments. At the time of surgery, they weighed a mean of 41 ± 5.5 kg each. They were fed high-calorie feed and allowed water ad lib. The protocol was submitted to the ethical board of animal investigations (Madrid, Spain) for the approval of animal experiments, and approval was received.

Surgical Preparation and Anesthesia

The animals were premedicated with intramuscular injections of midazolam (0.05 mg/kg body weight, Dormicum; Roche, Madrid, Spain); atropine (0.020 mg/kg body weight, Sulfato de Atropina; Servicios Farmaceuticos de la Defensa, Cordoba, Spain); carazolol (1 mL/50 kg body Weight, Suacron; Bayer Farmaceutica, Barcelona, Spain); and azaperon (0.25 to 1 mg/kg body weight, Stresnil; Esteve Farma, Barcelona, Spain).

For induction of anesthesia a funnel mask was used. Once the animal was in sufficiently deep anesthesia, an endotracheal tube was inserted and anesthesia was maintained by inhalation of oxygen, nitrous oxide, and isoflurane. Electrocardiographic monitoring was continued throughout the procedure. Surgery was done under aseptic conditions.

Implant Placement

An extraoral approach through an incision at the inferior border of the mandible was used to expose the facial wall of the mandibular body. Two implants/animal with a length of 4 mm and a diameter of 3.75 mm (Flange Fixture, Nobel Biocare, Göteborg, Sweden) were placed above the inferior border of the mandibular body (Figs 1a and 1b). One of them was placed without PRGF and served as a control, and the other one was placed with 0.4 mL with PRGF. One implant was placed cranially, and the other one was caudal to the first. Before application, PRGF was brought to room temperature to liquefy it and was applied locally into the ready drill hole with a sterile syringe immediately before implant placement. With the implants and the PRGF in place, the wound was sutured in layers.

Fig 1a Flange implant and drill.

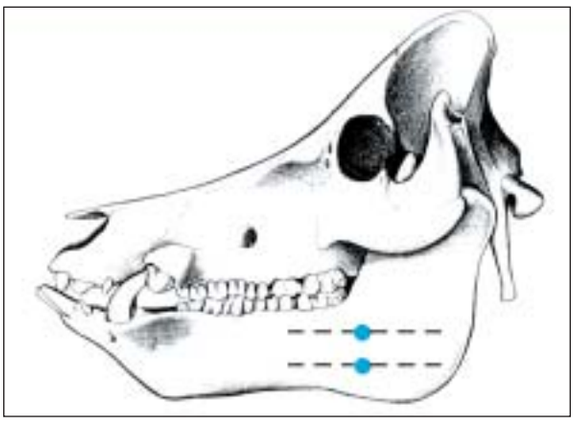

Fig 1b Schematic showing facial view of minipig mandible. Note the 2 dental implants. Saw cuts are indicated by interrupted lines.

Postoperative Medication and Follow-up

To prevent infections, the animals received amoxicillin (Clamoxyl; Pfizer, Madrid, Spain), 1.5 g intramuscularly, as a single dose postoperatively and butorfanol (Tubugesic; Fort Dodge Laboratories, Girona, Spain), 0.1 mg/kg body weight, for pain relief. Four animals were sacrificed at 4 weeks with a lethal anesthetic dose and the remaining 4 were sacrificed at 8 weeks.

Histology and Histomorphometry

The mandibles were removed 4 and 8 weeks after surgery, stripped of soft tissues, and fixed in buffered (neutral) 4% formalin solution. The region of interest was divided into several blocks parallel to the long axis of the implants. These blocks were dehydrated in ascending grades of alcohol and embedded in light-curing resin (Technovit 7200 VLC + BPO; Kulzer, Wehrheim, Germany). Using the Exakt cutting and grinding equipment (Exact Apparatebau, Norderstedt, Germany), the implant-bearing blocks were cut along the long axis of the implant and reduced to a thickness of 30 µm. The undecalcified cut and ground specimens thus obtained were stained with the Levai-Laczko stain.[21]

For histomorphometric evaluation, the specimens of 14 implants were photographed with a digital camera (Kodak Professional DCS 420; Eastman Kodak Company, Rochester, NY) mounted on a microscope (Nikon Microphot-FXA; Nikon Corporation, Tokyo, Japan). The resolution was 1 pixel = 3.4 µm. Then the entire region containing the implant with the surrounding tissues was digitized, and the contours of the implant, the local host bone, and the newly formed bone were retraced semimanually. On a personal computer, the percent contact length between the local host bone, the newly formed bone, and the implant of one representative section per implant was computed with the Lucia G 4.51 software (Laboratory Imaging, Brno, Czech Republic).

Statistical Analysis

Cell culture data were expressed as means of quadruple runs ± standard deviations. Differences were evaluated by the Student *t* test and $P \leq .05$ was considered significant.

To evaluate differences between the groups with and without PRGF, blocked analysis of variance was used, with 1 animal representing 1 block. Other predictive factors, ie, mandibular site and implanted region, were also considered, and interactions between these and the treatment were evaluated. Four-week and 8-week estimates were computed as least square means ± standard deviations.

For statistical analysis the SAS software package was used (SAS Institute, Cary, NC). All tests were 2-tailed and $P < .05$ was considered significant.

RESULTS

Mitogenic Activity of PRGF and Deep-Frozen Thawed PRGF

PRGF enhanced the ^{3}[H]-thymidine incorporation in gingival fibroblasts. The increase in the proliferation rate was dose-dependent. There was no significant difference between freshly prepared PRGF and PRGF that had been frozen in liquid nitrogen and then thawed (Table 1).

Table 1 Effects of Freezing PRGF on the Mitogenic Activity of Gingival Fibroblasts

	Dilution I	Dilution II	Dilution III	Control
PRGF	19.8 ± 1.6	5.8 ± .08	1.6 ± 0.6	1.0 ± 0.2
Deep-frozen thawed PRGF	21.2 ± 1.3 (ns)	6.6 ± 0.8 (ns)	1.6 ± 0.5 (ns)	1.0 ± 0.2

Experiments were performed as described in the Methods section. Results are expressed as x-fold stimulation of ^{3}[H]-thymidine incorporation versus untreated controls. Data are means ± SD of quadruples. ns = not significant versus fresh PRGF.

Fig 2a Longitudinal section through implant placed with PRGF after 4 weeks of healing time (Levai-Laczko; magnification ×5). Asterisk = host bone; arrowhead = newly formed bone.

Fig 2b Longitudinal section through control implant after 4 weeks of healing time (Levai-Laczko; magnification ×5). Asterisk = host bone; arrowhead = newly formed bone.

Clinical Course

All animals made a rapid postoperative recovery. Healing was uneventful throughout.

Histology

Implants With PRGF. The implants, the local host bone, and the newly formed bone were well defined in the histologic sections. Implants were seen to be in direct contact with the original local bone and the newly formed bone. Endosteal and periosteal osteoneogenesis increased over time. No cellular inflammatory reaction to allogenic PRGF was found (Fig 2a).

Implants Without PRGF. On histologic examination, the implants, the local host bone, and the newly formed bone were well distinguishable. The implant surfaces were in direct contact with the original local bone and the newly formed bone. Osteoneogenesis was mainly endosteal and periosteal. The amount of newly formed bone increased between weeks 4 and 8 (Fig 2b).

Histomorphometry

Overall, 55.30 ± 5.49% BIC was found in the PRGF group versus 38.91 ± 4.09% in the control (ie, no PRGF) group (P = .0198). At 4 weeks, BIC was 44.20 ± 5.40% with PRGF and 29.62 ± 5.40% without PRGF (P = .0632). At 8 weeks, percentages were 70.36 ± 13.21% with and 48.20 ± 6.73% without PRGF (P = .1221) (Figs 3a and 3b). The effects of the implant site were the same in the 2 groups (PRGF and controls). Implants placed caudally showed more BIC than those placed cranially.

DISCUSSION

In the present study, the effect of PRGF on BIC in minipig cortical bone was investigated. Platelet-released growth factors from allogenic sources was used to avoid interdonor variability of the growth factor concentration and composition.[22,23] The reason why no adverse reactions to allogenic PRGF were seen may be that allogenic PRGF does not contain platelet membranes, which carry immunogenic structures on their surface structures that may cause adverse reactions.[24]

The PRGF used in this study is mitogenic and can be stored without loss of biologic activity. This technique could be helpful clinically in that it would reduce the operating time and patient discomfort associated with numerous blood donations. Another

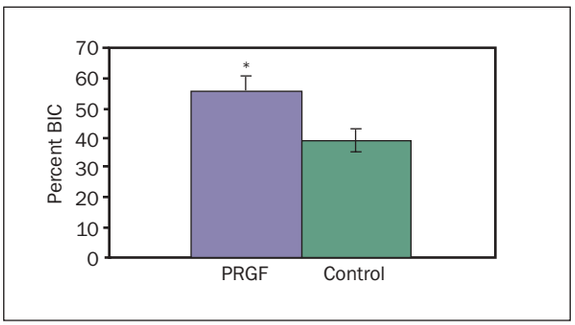

Fig 3a Histogram illustrating BIC with PRGF and without PRGF (controls) for the entire follow-up time, expressed as least square means. Histomorphometry indicated a significant difference between the groups. *P = .0198.

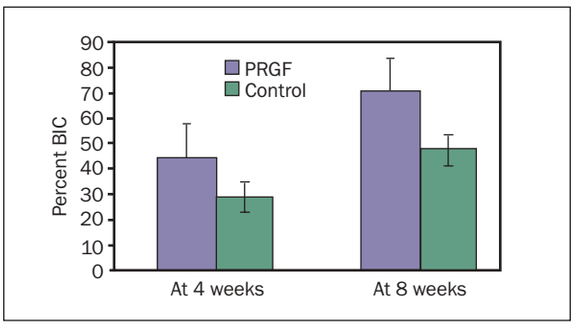

Fig 3b Histogram illustrating BIC with PRGF and without PRGF (controls) at 4 and at 8 weeks, expressed as least square means. The difference between the groups was P = .0632 at 4 weeks and P = .1221 at 8 weeks.

advantage would be that the quality of PRGF could be controlled without any time limit between blood sampling and surgery. With currently available PRP kits, PRP can be prepared immediately before surgery. Although quality control is limited,[19,22,23,25,26] presurgical preparation of the PRP would be highly desirable because of the variability of the growth factor content and their biologic activity.[22,23,25,26]

Minipigs are very similar to the human body in terms of platelet count, clotting parameters, and bone structure.[27] The body of the minipig mandible is also very similar to that in humans and contains mainly cortical bone.[18,28] The extraoral approach used for implant placement enabled the animals to feed more or less freely after surgery. It also was intended to prevent wound contamination by intraoral microorganisms.

In the present experiments, 55.30% BIC was found with PRGF versus 38.91% without PRGF. Lynch and coworkers reported 26.94% BIC 3 weeks after implant placement with recombinant PDGF and insulin-like growth factor-1 versus 34.27% without growth factors.[29] These discrepancies may have several reasons: the species used (dogs vs minipigs), the follow-up times (1 and 3 weeks versus 4 and 8 weeks), implant loading by masticatory forces during healing, and the type of growth factors used (recombinant versus natural). In contrast, in the present study, a mixture of natural growth factors as described by Marx and associates was used.[12]

In the current study, the effect of PRGF on BIC was not significant at 4 and at 8 weeks. But since BIC was increased by PRGF at both time points, it seemed legitimate to combine the data from both time points to obtain a larger, statistically more conclusive sample material. Throughout the entire follow-up time, BIC was significantly increased after implant placement with PRGF. Although cortical bone—known to have poor regenerative potential—was studied, the results concur with those of other studies on cancellous bone.[6–10] It can thus be speculated that the effects of PRGF in cortical bone are similar to those in cancellous bone.

CONCLUSION

Implants placed with PRGF showed increased BIC in cortical bone of minipigs in the animal model studied. These data indicate that, like PRP, the growth factors released from activated platelets can enhance the osseointegration of dental implants.

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