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Effect of platelet-released growth factors and collagen type I on osseous regeneration of mandibular defects

A pilot study in minipigs

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Abstract

Objective: To study the effects of platelet-released growth factors (PRGF) and collagen type I on bone defect healing in minipig mandibles.

Material and Methods: In eight adult minipigs defects were trephined in the facial mandibular wall from extra-oral and filled with collagen+PRGF or with collagen alone. Control defects were left untreated. PRGF were defined as the supernatants obtained after centrifugation of washed, thrombin-activated allogenic cells of platelet-rich plasma. The animals were sacrificed at 4 and 8 weeks. For histological analysis, undecalcified ground specimens stained with the Levai–Laczko stain were used.

Results: For the entire follow-up, the amount of newly formed bone was $35.49 \pm 3.84\%$ in the collagen+PRGF group, $46.34 \pm 3.84\%$ in the collagen-only group and $33.83 \pm 4.11\%$ in the controls. The differences between the collagen+PRGF and the collagen-only group ($p = 0.0343$), and between the collagen-only group and the controls ($p = 0.0305$) were significant. Histologically, defects filled with collagen+PRGF showed inflammatory reactions at 4 weeks, and new bone formation near the remnants of the filler collagen was reduced.

Conclusion: The data suggest that collagen type I alone, but not its combination with PRGF can support the early stages of cortical bone repair.

Key words: bone regeneration; collagen type I; mandible; minipigs; platelet-released growth factors (PRGF)

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In tissue injuries, platelets contribute to hemostasis and wound healing (Mannaioni et al. 1997). They are activated by thrombin and collagen and release a large number of growth factors such as platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β) and vascular endothelial growth factor (VEGF) at the injury site (Möhle et al. 1997, Marx et al. 1998, Gemmel & Park 2000). In the present study, platelet-released growth factors (PRGF) were defined as supernatants obtained after centrifugation of washed, thrombin-activated cells of platelet-rich plasma (PRP) (Gruber et al. 2002, Fuerst et al. 2004). Proliferation of bone, periosteum-derived, osteoclast-like cells and gingival fibroblasts is increased by PRGF (Gruber et al. 2002, 2003a, b, Fürst et al. 2003b). Healing of periodontal defects and bone regeneration was reported to be improved by thrombin-activated PRP (Camargo et al. 2002, Fennis et al. 2002).

In previous studies, PRP was used together with autogenous bone (Marx et al. 1998, Fennis et al. 2002). Autogenous bone is osteo-inductive and osteoconductive and therefore considered to be ''the golden standard'' (Bauer & Smith 2002). Harvesting autogenous bone causes donor site morbidity and additional cost (Younger & Chapman 1989). Therefore, there is a need to develop a clinical alternative to autogenous bone.

Collagen type I fulfills some criteria expected of a promotor of bone formation (Bauer & Smith 2002). It stimulates osteoblast proliferation and osteogenic differentiation of bone marrow-derived cells (Masi et al. 1992, Kinoshita et al. 1999). It is chemotactic for osteoblasts, fibroblasts and endothelial cells (Mundy et al. 1982, Palmieri et al. 2000). Collagen

type I has been used for filling empty sockets, periodontal fenestrations and bone defects by some researchers (Mannai et al. 1986, Choi et al. 1993, Güngörmüs & Kaya 2002). Others did not find any evidence of enhanced osteoneogenesis by collagen (Cook et al. 1994).

In this pilot study, the hypothesis to be tested was that bone formation would be increased by the combination of PRGF and collagen type I, which are both known to promote bone regeneration. The effects of PRGF and collagen type I on bone formation in the facial wall of minipig mandibles were examined by histomorphometric analysis after 4 and 8 weeks.

Material and Methods

Preparation of PRGF

PRP (about 30 ± 5 ml) from two minipigs was prepared by standard apheresis using a centrifuge (Cryofuge 6000®, Heraus Sepatech, Düsseldorf, Germany) and a triple blood bag system (Teruflex®, Terumo Europe, Leuven, Belgium) filled with 63 ml of CPD (citrated phosphate dextrose) (Fürst et al. 2003a).

Platelet-rich plasma cells were washed in Tyrode's buffer, pH 6.4, and centrifuged at $1400 \times g$ for 10 min. Pellets were resuspended in DMEM/F12 medium (Life Technologies, Grand Island, NY, USA) resulting in platelet counts of 2×10^9/ml. The release of platelet factors into the supernatant was induced by adding human thrombin, 2 IU/ml (Tissucol®, Baxter, Vienna, Austria) for 30 min at room temperature. After centrifugation at $1400 \times g$ for 10 min, the supernatant termed PRGF was collected and stored at -40°C (Fuerst et al. 2004).

Animals

Eight adult miniature pigs (4 males and 4 females bred from Minnesota Pigs and Vietnamese Pot-bellied Pigs) were used. At the time of surgery they weighed 41 ± 5.5 kg. The animals were kept on high-calorie feed and allowed water ad lib. The protocol was approved by the ethical board of animal investigations (Madrid, Spain) for the approval of animal experiments.

Surgical prepping and anesthesia

All animals were premedicated with midazolam, 0.05 mg/kg body weight (Dormicum®, Roche Madrid, Spain), atropin, 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) by i.m. injection.

For induction of anesthesia a funnel mask was used. At an adequate depth of anesthesia an endotracheal tube was inserted and anesthesia was maintained with O_2, N_2O and isoflurane by inhalation. Surgery was done with due attention to aseptical precautions and under continuous ECG monitoring.

Surgical procedure

Through an extra-oral incision at the lower border of the mandible the facial aspect of the mandibular body was exposed and defects of 6 mM depth were drilled with an 8-mM trephine. The defects were approximately 5–7 mM away from the lower border of the mandible and spaced at an intercavity distance of approximately 8 mM. (Fig. 1) They were filled at random with either collagen type I (Lyostypt®, Braun, Austria)+PRGF or with collagen type I alone. Control defects were left untreated. The wounds were sutured in layers. PRGF was thawed immediately before surgery. Collagen type I was incubated with either DMEM /F12 medium or PRGF, both in excess, for 15 min.

Postoperative medication

To prevent infections all animals received a single i.m. dose of amoxicillin (Clamoxyl®, Pfizer, Madrid, Spain), 1.5 g, postoperatively. Butorfanol (Torbugesic®, Fort Dodge Laboratories, Girona, Spain), 0.1 mg/kg body weight i.v., was administered for pain relief.

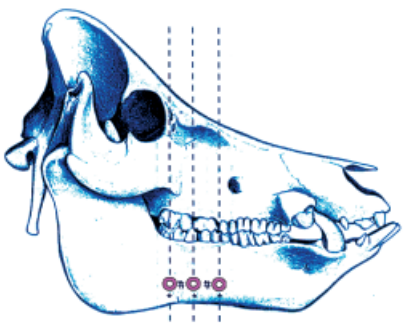

Fig. 1. Schematic illustrating the facial view of the minipig mandible. Note three cavities. Saw cuts marked by interrupted lines. #, distance between two cavities = 8–10 mM. *, distance from lower border of mandible = 5–7 mm.

Follow-up

Four animals were sacrificed at 4 weeks and the remaining 4 at 8 weeks post surgery by injection of a lethal anesthetic dose.

Histology and histomorphometry

Four and 8 weeks post surgery, the mandibles were removed, scraped clean of soft tissue and fixed in buffered (neutral) 4% formalin solution. The region of interest was divided into several blocks by saw cuts parallel to the trephined holes (Fig. 1). The blocks were dehydrated in ascending grades of alcohol and embedded in light-curing resin (Technovit 7200 VLC+BPO; Kulzer and Co., Wehrheim, Germany). For further processing, the Exakt Cutting and Grinding equipment (Exakt Apparatebau, Norderstedt, Germany) was used. With it the blocks containing one trephined hole each were divided along the long axis of the holes and reduced to a thickness of 30 μm. The undecalcified cut and ground sections (one of each cavity) were stained with the Levai–Laczko stain (Donath, 1988).

For histomorphometric analysis, photographs of four specimens of each treatment option per time point were made with a digital camera (Kodak Professional DCS 420; Eastman Kodak Company, Rochester, NY, USA) mounted on a microscope (Nikon Microphot-FXA, Nikon Corporation, Tokyo, Japan) at a resolution of 1 pixel equal to 3.4 μm. The region of interest including the drill holes and the surrounding structures was digitized. (Fig. 2a) The contours of the original local and the newly formed bone were outlined semimanually. The drill holes were divided into four equal areas: Two of them were defined as the center and two of them as the periphery. To analyze the effect of PRGF and collagen type I on bone formation the newly formed bone in the periphery, which had contact with the cavity margin, was evaluated. The newly formed bone on the facial aspect of the mandible between the cavities was not included in the measurement, because bone regeneration varied widely as a function of intra-operative periosteal stripping at this site. The percent

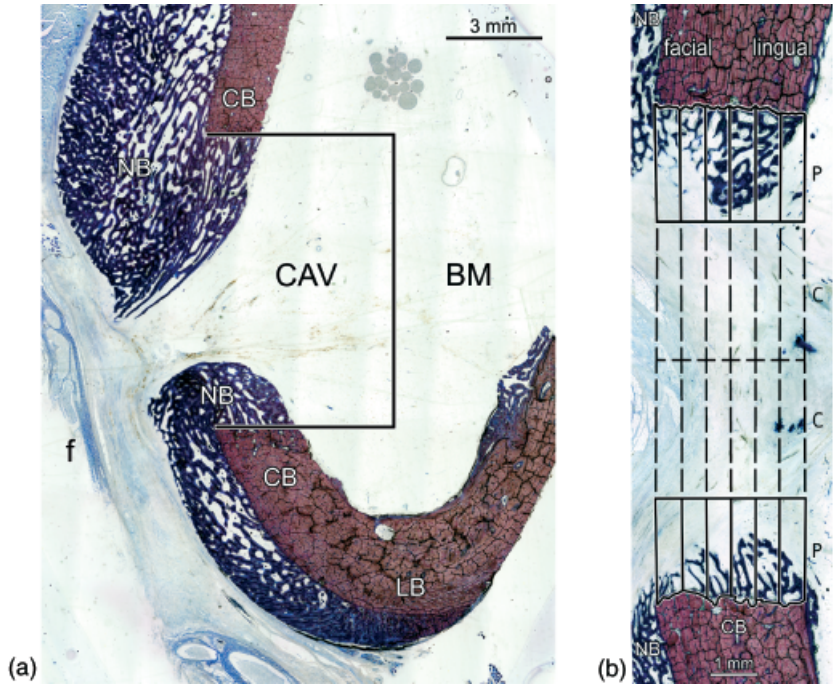

Fig. 2. (a) Section made by cutting perpendicularly to the facial aspect of the minipig mandible. Levai–Laczko stain. NB, new bone; CB, cortical bone; LB, lower border of the mandible; BM, bone marrow; f, facial; C, cavity. (b). Section through facial wall of the mandible. Levai–Laczko stain. The histomorphometric image was subdivided in fields of 2 mM × 0.5 mM. To calculate the percent newly formed bone, the measured area of newly formed bone was divided by the total area measured and multiplied by 100. The total area measured consisted of newly formed bone and non-mineralized soft tissue. NB, new bone; CB, cortical bone; P, periphery; C, center.

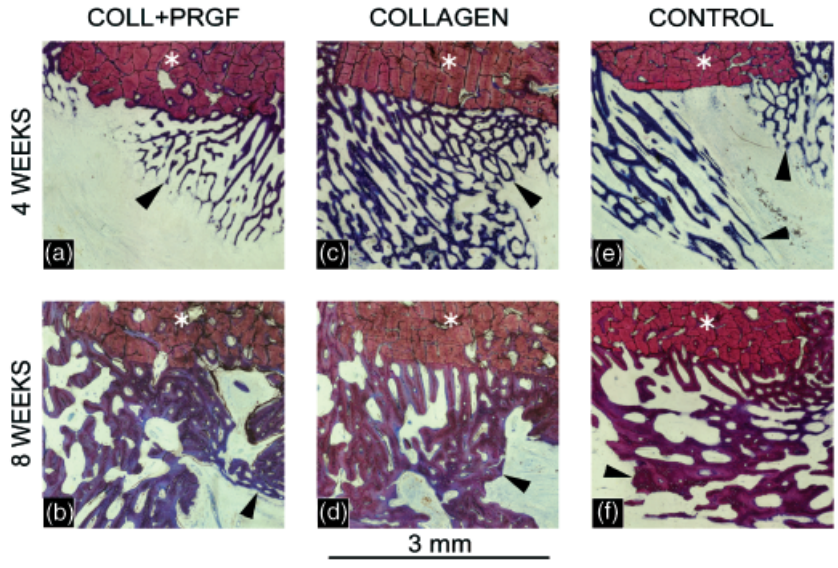

Fig. 3. Sections through the facial wall of the mandible. Levai–Laczko stain. Newly formed bone marked by triangles und cortical bone by asterisks. Facial defect margins are on the left side. (a) Cavity filled with collagen+PRGF at 4 weeks; (b) cavity filled with collagen+PRGF at 8 weeks; (c) cavity filled with collagen alone at 4 weeks; (d) cavity filled with collagen alone at 8 weeks; (e) control cavity at 4 weeks; (f) control cavity at 8 weeks. At 4 weeks, more bone formation was present in the collagen group than in the controls. No difference in bone formation was seen between collagen+PRGF and the controls at the same time point. The amount of newly formed bone was similar in all groups at 8 weeks.

newly formed bone was computed on a personal computer with the Lucia G 4.51 software (Laboratory Imaging Ltd, Brno, Czech Republic) within 2 mM of the defect margins. Histomorphometric images were subdivided in fields of 2 mM × 0.5 mM. (Fig. 2b) To calculate the percent newly formed bone, the measured area of newly formed bone was divided by the total area measured and multiplied by 100. The total area measured consisted of newly formed bone and non-mineralized soft tissue (Fig. 2b).

Statistical analysis

Histomorphometric data were obtained at 4 and 8 weeks each with four animals. Every animal had three defects with different fillings (control, collagen alone and collagen+PRGF) located either mesial or distal. Each defect was measured at two different positions – peripheral and central. So each animal contributed six measurements, except one animal that had only two fillings. Associations between histomorphometric measurements and healing interval, type of filling, location and measured region were calculated by analysis of variance with repeated measurements and interactions between prognostic factors were also investigated. Least-square means and corresponding standard errors of the mean were used to describe the data. Statistical significance was computed with the SAS® Statistics software package (SAS Institute Inc., Version 8, Cary, NC, USA). All tests were two-tailed and $p < 0.05$ was considered significant.

Results

Clinical course

Postoperative healing was uneventful throughout.

Histology

Collagen+PRGF

At four weeks none of the defects was obliterated by bone. Along the margins new bone was present mostly at the periosteal side. New bone grew into the defects and toward the cortical margins. Quantitatively, little woven bone had been formed. (Fig. 3a) Collagenous tissue with a high fiber content was present in the center of the defects. This contained multinucleated macrophages in the vicinity of the filler collagen.

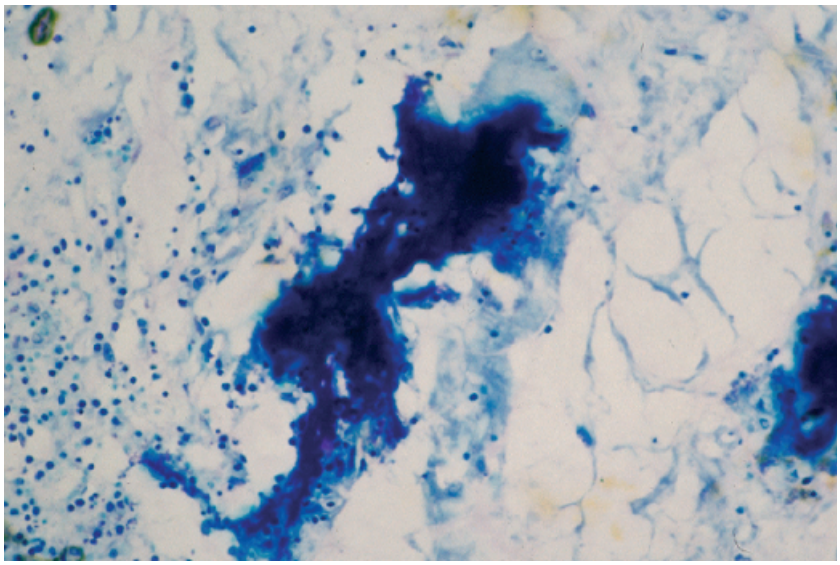

Fig. 4. Cavity filled with collagen+PRGF at 4 weeks post surgery. In the center of the cavity collagenous tissue with a high fiber content was present. This contained multinucleated macrophages in the vicinity of the filler collagen. Osteoneogensis was clearly reduced around the filler collagen and the macrophages. Levai–Laczko stain, × 50.

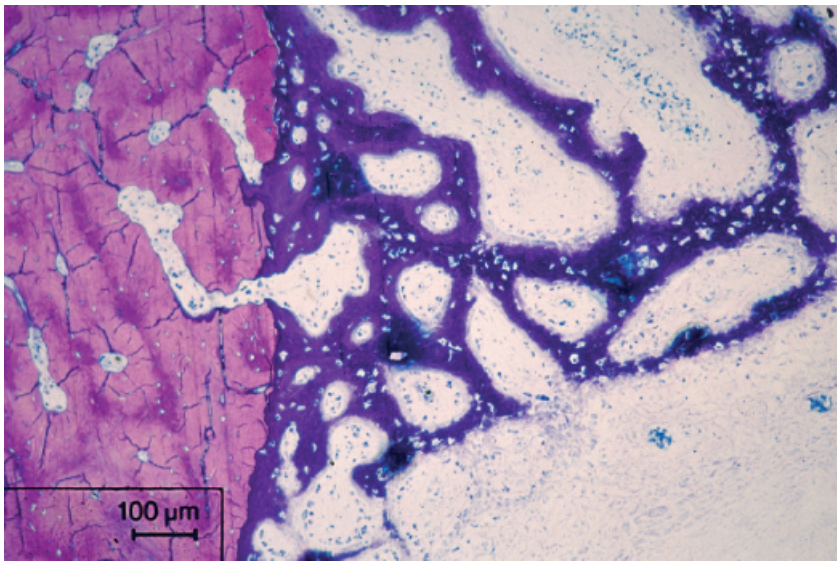

Fig. 5. Cavity filled with collagen alone at 4 weeks post surgery. At the margins of the defects Haversian canals were dilated by bone resorption and connected with the newly formed bone by cutting cones. Levai–Laczko stain, × 50.

Osteoneogensis was clearly reduced around the filler collagen and the macrophages (Fig. 4).

At 8 weeks, the defects were partly obliterated by bone. The newly formed bone was lamellar in nature and immature. At the margins of the defects the bone was compacted (Fig. 3b).

Collagen

Four weeks post surgery newly formed bone originating from the periosteum, endosteum and the defect margins had not completely obliterated the defects. Newly formed bone was lamellar in nature and immature (Fig. 3c). At the margins of the defects Haversian canals were dilated by bone resorption and connected with the newly formed bone by cutting cones (Fig. 5). Some filler collagen was still interspersed between the newly formed bone. Multinucleated macrophages were sparse.

At 8 weeks most of the defects were obliterated by newly formed bone (Fig. 3d). Woven bone was less prominent and had mostly been remodeled to lamellar bone. The trabeculae were thickened and the cancellous spaces were thin. Medullary spaces occupied less of the bone mass than at 4 weeks.

Controls

At 4 weeks, the margins of the trephined holes in the cortical bone of the mandible were clearly visible histologically. Bone repair originated from the marginal periosteum and endosteum and advanced toward the center of the defects in a spur-like pattern. Complete bony union was absent (Fig. 3e). Haversian canals extending from the original local into the newly formed bone signaled bone remodeling at the margins.

At 8 weeks the defects had become smaller, but were not completely obliterated. The newly formed bone showed signs of remodeling from woven to lamellar bone (Fig. 3f). At the margins of the defects Haversian canals from the local bone entered the newly formed bone signaling further remodeling.

Histomorphometry

For the entire follow-up, the percent of newly formed bone was 35.49 ± 3.84 in the collagen+PRGF group, 46.34 ± 3.84 in the collagen-only group and 33.83 ± 4.11 in the control group. The difference between the collagen+PRGF and the collagen-only group was significant ($p = 0.0343$), that between the collagen+PRGF group and the control group was not significant ($p = 0.7638$), but that between the collagen group and the control group was again significant ($p = 0.0305$) (Fig. 6).

At 4 weeks, $17.67 \pm 5.24\%$ new bone had been formed in defects with collagen+PRGF versus $29.89 \pm 5.24\%$ in defects with collagen alone and $13.40\% \pm 5.24\%$ in the untreated controls. While no significant differences were found between collagen+PRGF and collagen alone ($p = 0.0870$) and between collagen+PRGF and the controls ($p = 0.5390$), the difference between the collagen group and the controls was significant ($p = 0.0237$).

At 8 weeks $53.32 \pm 5.62\%$ new bone had been formed in the holes with collagen+PRGF versus $62.80 \pm 5.62\%$ in the holes with collagen alone and $54.26 \pm 6.35\%$ in the controls. No significant differences were found between collagen+PRGF and collagen

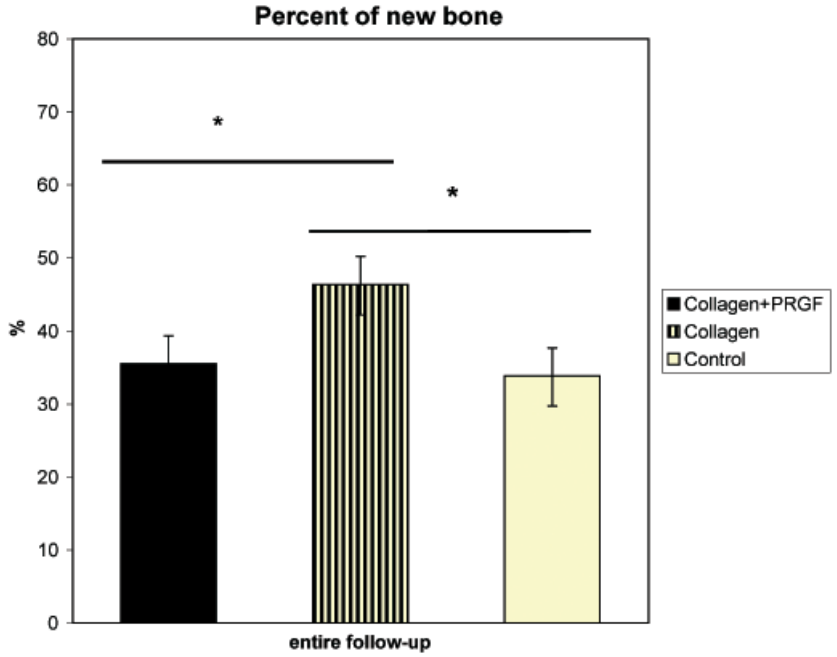

Fig. 6. Histogram illustrating the percent newly formed bone for the entire follow-up after collagen+PRGF or collagen-alone filling and in controls. Least-squares means and standard errors of the mean. The difference between the collagen+PRGF and the collagen-only group was $p = 0.0343$, that between the collagen group and the controls $p = 0.0305$. $^*p < 0.05$.

Table 1. Histomorphometric data following defect filling with collagen+PRGF (platelet-released growth factor), collagen alone and controls expressed as least-square means and corresponding standard errors of the mean

	4 weeks (%)	8 weeks (%)
collagen+PRGF	17.67 ± 5.24	53.32 ± 5.62
collagen	29.89 ± 5.24*	62.80 ± 5.62
controls	13.40 ± 5.24	54.26 ± 6.35

At 4 weeks, the difference between the collagen group and the controls was significant. No significant differences were found between collagen+PRGF and collagen alone and between collagen+PRGF and the controls. At 8 weeks, no significant differences were detected between the groups.
*$p = 0.0237$ versus controls.

alone ($p = 0.1797$), between collagen+PRGF and the controls ($p = 0.9125$), and between collagen alone and the controls ($p = 0.3259$) (Table 1).

Discussion

In this pilot study new bone formation in mandibular defects filled with collagen+PRGF or with collagen alone was investigated in minipigs.

PRGF have been shown to be mitogenic for periosteum-derived and bone cells in vitro and to increase bone-to-implant contacts in the minipig mandible (Gruber et al. 2002, 2003b, Fuerst et al. 2003b). In this pilot study, PRGF from pooled allogenic PRP was used to avoid interdonor variability of growth factor content and composition (Appel et al. 2002, Weibrich et al. 2003). PRGF do not contain platelet membranes, which carry immunogenic structures that are responsible for adverse reactions (Rozman, 2002). In previous studies, neither xenogenic PRP nor allogenic PRGF impaired bone formation or caused adverse reactions (Kania et al. 1998, Fuerst et al. 2004). In agreement with the reported observations allogenic PRGF did not cause adverse reactions in the present animal study.

PRGF can be prepared prior to surgery and deep-frozen for storage. This may be an advantage clinically, versus PRP. Current recommendations are that PRP should be prepared within 6 hours from surgery for immediate use (Marx et al. 1998; Sanchez et al. 2003). In addition, both their cell counts and growth factor concentrations vary widely (Weibrich et al. 2003). In clinical routine the growth factor content and the mitogenic activity of PRP preparations cannot be re-evaluated immediately before surgery (Sanchez et al. 2003). PRGF, by contrast, can be prepared at any time irrespective of the date of surgery. As a result, its quality can easily be re-evaluated between blood sampling and surgery.

Minipigs are commonly used for studying bone regeneration and evaluating the healing characteristics of dental implants (Buser et al. 1998, Schliephake et al. 1998, Terheyden et al. 1999, Zechner et al. 2003). Our histologic findings indicated that newly formed bone was mainly derived from the periosteum. Only some bone originated from the endosteum and the defect margins, while none was derived from the mandibular canal. The reason may be that abundant cortical bone at the defect margins and non-osseous tissue in the mandibular canal were present at the surgical site. Both contain few potential bone-forming cells and may therefore have a low regenerative potential (Buser et al. 1998, Schenk & Buser 1998, Schliephake et al. 1998, Fuerst et al. 2004). This made the minipig mandible used in our experiments an ideal candidate for studying new bone formation in slowly healing bone.

In this pilot study the amount of newly formed bone in the collagen+PRGF group was significantly reduced compared to the collagen-only group. No significantly different behavior was observed between the groups at 4 and 8 weeks, nor were there significant differences between 4 and 8 weeks, mainly because of the small sample size at 4 and 8 weeks. Also, bone formation was reduced around remnants of the filler collagen and around macrophages at 4 weeks. This may be explained by the multiple mechanisms of PRGF, which contribute to osteoclastogenesis and are chemotactic for leucocytes (Mannaioni et al. 1997, Gruber et al. 2002). In agreement with the present findings, Selvig et al. (1994) surmised that filler collagen in combination with TGF-β, which is also highly concentrated in PRGF, prolonged the activity of macrophages so that catabolic processes can gain ascendancy.

Like other in vitro and in vivo studies, the present experiments failed to provide evidence of an increased osteogenesis

by PRGF in combination with collagen. The clinical use of this regimen cannot therefore, be recommended without further evidence-based data.

Collagen type I significantly increased the amount of newly formed bone in this study. The increased osteoneogenesis may well be attributable to the stimulatory effect of collagen on local cells involved in bone regeneration such as osteoblasts, mesenchymal progenitor cells and endothelial cells (Masi et al. 1992, Kinoshita et al. 1999, Palmieri et al. 2000). The effect of collagen on bone formation was more pronounced at 4 weeks, and the difference versus the controls became less evident at 8 weeks. This may be attributable to the short-lived effect of filler collagen on new bone formation (Knowles et al. 1991, Schmitt et al. 1999). This assumption is supported by studies with a longer follow-up time of 12 weeks, which failed to show increased osteoneogenesis by collagen (Cook et al. 1994).

The data from the present pilot study suggest that the application of collagen type I may be beneficial for bone formation during the early phase of cortical bone healing. Admixing PRGF to it does not enhance this effect.

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