Chapter 7

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The effect of matrix bound parathyroid hormone on bone regeneration

Key words: bone regeneration, carrier material, dental implants, parathyroid hormone, polyethylene glycol

Abstract

Introduction: Autogenous bone is the most successful bone-grafting material; however, multiple disadvantages continue to drive developments of improved methods for bone regeneration.

Aim: The aim of the present study was to test the hypothesis that an arginine–glycine–aspartic acid (RGD) modified polyethylene glycol-based matrix (PEG) containing covalently bound peptides of the parathyroid hormone (PTH1–34) enhances bone regeneration to a degree similar to autogenous bone.

Material and methods: Six American foxhounds received a total of 48 cylindrical titanium implants placed in the mandible between the first premolar and the second molar. Five, respectively, 7 months following tooth extraction, implants were placed into the center of surgically created defects. This resulted in a circumferential bone defect simulating an alveolar defect with a circular gap of 1.5 mm. Four treatment modalities were randomly allocated to the four defects per side: (1) PEG-matrix containing 20 μg/ml of PTH1–34, and 350 μg/ml of G6R6 peptide, (2) PEG alone, (3) autogenous bone and (4) empty defects. Histomorphometric analysis was performed 4 and 12 weeks after implantation. The area fraction of newly formed bone was determined within the former defect and the degree of bone-to-implant contact (BIC) was evaluated both in the defect region and in the apical region of the implant. For statistical analysis ANOVA and subsequent pairwise Student’s t-test were applied.

Results: Healing was uneventful and all implants were histologically integrated. Histomorphometric analysis after 4 weeks showed an average area fraction of newly formed bone of 41.7 ± 1.8% for matrix-PTH, 26.6 ± 4.1% for PEG alone, 43.9 ± 4.5% for autogenous bone, and 28.9 ± 1.5% for empty defects. After 12 weeks, the respective values were 49.4 ± 7.0% for matrix-PTH, 39.3 ± 5.7% for PEG alone, 50.5 ± 3.4% for autogenous bone and 38.7 ± 1.9% for empty defects. Statistical analysis after 4 and 12 weeks revealed significantly more newly formed bone in the PTH1–34 group compared with PEG alone or empty defects, whereas no difference could be detected against autogenous bone. Regarding BIC no significant difference was observed between the four treatment groups neither at 4 nor at 12 weeks.

Conclusion: It is concluded that an RGD-modified PEG hydrogel containing PTH1–34 is an effective matrix system to obtain bone regeneration.

Dental implant therapy has become a very predictable method rendering excellent implant survival rates [Berglundh et al. 2002]. In cases with insufficient amounts of bone the regeneration of bone tissue becomes a necessity. Autogenous bone is the most
successful bone-grafting material; however, limited availability and donor site morbidity continue to drive developments for optional methods for bone regeneration (Nkenke et al. 2001).

Basic science and clinical studies have documented the ability of different growth and differentiation factors to induce and enhance bone regeneration (Lynch et al. 1991; Cho et al. 1995; Jung et al. 2003). It has been shown that the regenerative potential of such factors depends on a high degree on the method of application (Sigurdsson et al. 1996). The use of biomaterials that allow sustained release of growth factors greatly enhances efficacy and allows reducing the protein dosage. In a recent in vivo and in vitro study it could be demonstrated that the release kinetics of bone morphogenetic protein/rhBMP-2 can be controlled by entrapping the rhBMP-2 into a biomimetic synthetic matrix made of polyethylene glycol (PEG). This resulted in an efficient bone regeneration resolving localized bone defects (Lutolf et al. 2003).

In addition to growth factors, the use of peptide fragment of the parathyroid hormone (PTH) have also been investigated for modulating bone healing. The 34 amino acid domain of the PTH has been shown to have multiple anabolic effects on both cancellous and cortical bone (Rizzoli et al. 1992; Whitlefield et al. 2000). Presently, this PTH1-34 peptide is used for systemic treatment of osteoporosis. Clinical trials have shown this therapy to be effective in increasing bone density (Neces et al. 2001). While PTH1-34 has a substantial effect in the treatment of osteoporosis, its use for the healing of local bone defects by using an activated matrix system has not yet been evaluated.

The aim of the present study was to test the hypothesis that a synthetic, arginine-glycine-aspartic acid (RGD) modified PEG containing a covalently bound peptide of the PTH1-34 enhances bone regeneration to a degree comparable with autogenous bone.

Material and methods

Synthetic matrix and bioactive peptides

The synthetic matrix used in the present study was a PEG-based hydrogel formed by combining a four-arm PEG with acrylate endgroups (Nektar Therapeutics, Huntsville, AL, USA; Elbert et al. 2001). In an aqueous buffer system (triethanolamine/HCl) the PEG termini connected through a highly self-selective addition reaction thus, forming an elastic PEG gel network within a few minutes. After mixing the four-arm PEG-acrylate solution with the PEG-dithiol solution, the gelling mixture was then drawn up into a sterile syringe with blunt needle and handed to the surgeon for application.

For preparing the activated gels the PEG-acrylate solution was supplemented by a 35 amino acid peptide of parathyroid hormone (cys-PTH1-34) and a 9 amino acid cys-RGD peptide. The thiol groups on the cystein moieties of these peptides were allowed to react with the PEG-acrylate before addition of the PEG-dithiol, facilitating their covalent incorporation into the gels (Lutolf et al. 2001; Fittkauf et al. 2005). This resulted in final concentrations of 20 µg/ml gels for cys-PTH1-34 and 350 µg/ml gels for cys-RGD.

In vitro gel degradation

In order to assess in vitro disintegration of the PEG gels, six cylindrical gels were kept at 37°C in 30 mM phosphate-buffered saline, pH 7.4. Each day for 13 days, the normalized weights of the dry-botted gels were assessed by dividing their weights by their equilibrated weights after 16 h. After 13 days, the gels were dissolved completely.

Surgical procedure

The current study protocol was approved by the University of Texas Health Science Center at San Antonio Institutional Use and Care of Animals Committee. In six males, laboratory-bred American foxhounds mandibular premolars and first molars were extracted. Five and 7 months after tooth extraction, implantation and regenerative surgery were performed. In order to have two time points and not to increase the number of animals two operations have been performed per animal each on one side. In the total of six animals, four implants were placed 3 months after tooth extraction in one side of the mandible and four implants after 7 months in the other side of the mandible. The animals were sacrificed 4 weeks after the last surgery. This provided healing time points, where both early (4 weeks) and late events (12 weeks) could be observed.

Following crestal incisions full-thickness flaps were elevated in one side of the mandible. Surgical preparation and implant placement at 4 experimental sites were performed on each side of the mandible according to a previously published protocol (Cochran et al. 1999). In brief, the implants were cylindrical with a large grit sandblasted and acid-etched (SLA) surface (Institut Straumann AG, Basel, Switzerland). They exhibited a screw design in the apical half only. The outer diameter without the threads measured 2.8 mm and the implant length 8 mm. A total of

![Fig. 1](image_url)  
**Fig. 1.** Diagram of implant in defect site after osteotomy preparation. The resulting histological defect was 1.5 mm wide and 4 mm deep on each side of the implant.
48 implants were placed into the center of the surgically created defect obtaining primary stability. This resulted in a circumferential bone defect simulating an alveolar defect with a circular gap of 1.5 mm [Fig. 1]. The four implants inserted per time point in each dog were randomly allocated to four treatment modalities:

1. PEG matrix containing 20 µg/ml of cys-PTH$_{1-34}$ and 1500 µg/ml cys-RGD peptide [matrix-PTH],
2. PEG alone,
3. Autogenous bone [positive control],
4. Empty defect [negative control].

At the matrix-PTH and the PEG alone sites, the PEG was applied by a syringe to fill the surgically created defects. Once placed into the defect the gel polymerized after approximately 40s.

The autogenous bone was harvested during the drilling process from each of the four defects and was used to augment the site determined for autogenous bone [Fig. 2]. Primary wound closure was achieved with interrupted sutures.

**Histological preparation and histomorphometric analysis**

Twelve weeks following the first regenerative surgery and 4 weeks after the second regenerative surgery, respectively, all six animals were sacrificed. The mandibles were resected and prepared for histological analysis.

The specimens were dehydrated and embedded in methylmethacrylate without being decalcified. Tissue blocks were cut exhaustively into 300-µm-thick vertical sections in the bucco-lingual plane using a slow-speed diamond saw (Leco Corporation, St Joseph, MI, USA). The sections were ground and polished to a final thickness of 100 µm and stained superficially with toluidine blue followed by basic fuchsin [Schenk et al. 1984].

From each specimen the central orofacial section through the implant was selected for quantitative assessment by applying standard morphometrical techniques (Weibel 1980; Gundersen et al. 1988). Measurements were carried out directly by light microscopy. The area fraction of newly formed bone within the former defects at the buccal and the lingual side of the implants were evaluated using an optically superimposed eyepiece test grid composed of 100 points and 10 cyclid lines at magnifications of ×40 [Schenk & Olah 1980]. The degree of BIC was evaluated both in the former defect region and in the region apical of original defect. The assessment in the former defect area was made from the most coronal level of BIC to the bottom of the former defect [Botticelli et al. 2004].

**Statistical analysis**

Mean values and standard deviations were calculated for the area fraction of newly formed bone within the former defect and for the BIC. For statistical analysis ANOVA and subsequent pairwise Student’s t-test were applied to detect differences between the four treatment modalities. Holm’s correction was used to account for multiple testing.

**Results**

**In vitro gel degradation**

*In vitro* hydrolytic degradation of the gels showed a progressive loss of weight until complete dissolution at day 13 [Fig. 3].

**Descriptive histology**

All 48 implants were osseointegrated after 4 and 12 weeks of healing. No biologically significant abnormality was observed in association with any of the treatment procedures. The staining allowed the distinction between preexisting and newly formed bone, as the preexisting bone was stained in a weaker color. Within the defects treated with autogenous bone no evidence of residual dual autogenous bone particles could be detected. In general, after 4 weeks, the lingual and the buccal side of all treated sites showed various amounts of apposition of newly formed trabecular bone to the implant surface with an endosteal bone in-growth. In the matrix-PTH group and in the autogenous bone group significantly more bone was observed around the implants [Fig. 4]. In addition, bone growth over the top of the implants was detected in one matrix-PTH treated site [Fig. 5] and one site treated with autogenous bone chips.

After 12 weeks of healing remodeling had occurred such that more mature and cortical bone was found in the former defect areas.

**Histomorphometric analysis**

The quantitative histomorphometric analysis revealed that both after 4 and
12 weeks the matrix-PTH group and the autogenous bone group demonstrated a higher area fraction of newly formed bone within the former defects. Average area fractions of newly formed bone after 4 weeks amounted to $28.9 \pm 1.5\%$ for the empty control group, to $43.9 \pm 4.3\%$ for the autogenous bone group, to $26.6 \pm 4.1\%$ for the PEG alone group, and to $41.7 \pm 1.8\%$ for the matrix-PTH group. After 12 weeks of healing the average area fractions of newly formed bone was a gain higher for the autogenous bone group ($50.5 \pm 3.4\%$) and for the matrix-PTH group ($49.4 \pm 7\%$) compared with the empty control group ($38.7 \pm 1.9\%$) and the PEG alone group ($39.3 \pm 5.7\%$) (Fig. 6).

Statistically significant differences in area fraction of newly formed bone within the former defect were observed for the matrix-PTH group compared with PEG alone or empty controls, whereas no difference could be detected against the autogenous bone group. The PEG alone showed no significant difference compared with empty defects. The differences between the groups were larger after 4 compared with 12 weeks of healing (Fig. 7). However, the area fraction of newly formed bone had significantly increased from 4 to 12 weeks for all treatment groups. The empty control showed the least bone area at both time points. Between 4 and 12 weeks, however, the highest bone gain was observed in the empty controls.

Regarding BIC no significant difference was observed between the four treatment groups neither at 4 nor at 12 weeks (Fig. 8).

Discussion

The present study demonstrated a clear beneficial effect on bone regeneration by use of a synthetic, RGD modified PEG containing a covalently bound peptide of PTH$_{1-34}$. This was documented by a similar amount of bone regeneration around dental implants compared with using autogenous bone.

In the past 75 years, a large number of studies have demonstrated that daily injections of small doses of PTH stimulate cortical bone growth, increase cortical bone strength, and increase trabecular thickness [Selye 1932; Brommage et al. 1999; Jerome et al. 1999] In addition, it has been shown that the peptide used in the present study has similar biological effects as the full 88 amino acid protein in activating osteoblasts and subsequent calcium release [Fujita et al. 1993]. The mechanism by which PTH influences bone remodeling is rather complex. PTH can increase
bone mass by expanding the activity and the number of osteoblasts presumably by preventing apoptosis and thus extending their working lifespan [Jilka et al. 1999]. Furthermore, the pharmacokinetics of PTH is of great importance. When PTH is administered by a pulsatile method bone density will increase. In contrast, when it is administered in a continuous manner, bone density will decrease most likely due to stimulation of osteoclast function [Kanatani et al. 1998].

Human trials have demonstrated anabolic effects of PTH treatment in both women and men with osteoporosis [Neer et al. 2001; Orwoll et al. 2003]. Daily subcutaneous administration of 20 μg PTH1–34 increased the total bone mineral density and decreased the risk of bone fractures [Neer et al. 2001]. Animal experiments have documented that intermittent injection of PTH enhances guided bone regeneration and increases mechanical strength and density of new bone after distraction osteogenesis [Andreasson & Cacciafesta 2004; Seebach et al. 2004]. Great advantages regarding dosage and patient comfort would result, if intermittent systemic injections of PTH could be replaced by sustained local release of the peptide to treat local bone defects. The PEG-based hydrogel used in the present study has been specifically designed for local presentation of the PTH. The PEG matrix has been optimized by the addition of pendant oligopeptide ligands for cell adhesion [RGD peptides; Yamada 1991]. It has been reported that primary human fibroblasts migrated radially out from clusters into the surrounding PEG matrix (Lutolf et al. 2003). Invasion was absent within networks lacking RGD ligand. Therefore, it can be expected that the presently used RGD modified PEG matrix may facilitate cell migration. In addition, it could be demonstrated in several experimental studies that implant surfaces, which have been biologically functionalized with RGD can increase bone formation compared with uncoated implants (Schaffner et al. 1999; Elmgaard et al. 2005; Schliephake et al. 2005). The possible combination of cell attraction by the RGD within the PEG matrix and the activation of the osteoblasts by the PTH may lead to a synergistic effect in the present study.

The in vitro evaluation in the present study revealed complete disintegration of the gels at day 13. This hydrolytic degradation of the network may facilitate cell infiltration and provide space for bone formation.

In the present study similar amounts of bone were found in the empty (28.9%) and the PEG alone (26.6%) groups after 4 weeks. These values are within the range (32.1%) reported in a previous animal study with a similar design [Botticelli et al. 2004]. In that study, however, no grafted materials were used but membranes were applied covering the defects. This indicates that the PEG alone did not improve bone regeneration when compared with spontaneous healing. A recent study using the same animal model applied collagen sponges loaded with 200 μg/ml rhBMP-2 [Cochran et al. 1999]. Significantly more bone formation was observed in the rhBMP-2-treated sites compared with the collagen-treated sites after 4 weeks. In the present study a similar significant increase could be achieved by using 20 μg/ml PTH compared with controls at 4 weeks. At 12 weeks the differences were smaller but still significant. These findings are also consistent with the results after 12 weeks with and without rhBMP-2, respectively [Cochran et al. 1999]. In the present study the BIC was similar in all groups after 3 months (65.5–67.3%). These values are comparable with the ones reported after 4 months of healing (65.6–69.9%) when a variety of defect dimensions were treated with membranes [Botticelli et al. 2004]. Neither matrix PTH nor autogenous bone was able
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to significantly increase BIC compared with PEG alone and empty defects. This might be due to the fact that no membranes were used in the present study, which could prevent soft tissue in-growth from the covering flap. In addition, no differences in terms of BIC were seen in the apical region, indicating that no effect from the treatment was transferred into the existing bone.

It is concluded that this synthetic, RGD modified PEG hydrogel containing covalently bound PTH1–34 is an effective matrix system to obtain bone regeneration in circumferential bone defects in the dog mandible. The present study demonstrated that with the use of this synthetic PEG matrix comparable levels of bone regeneration to the gold standard, autogenous bone chips, could be achieved. The PEG matrix alone did not ameliorate the bone regeneration when compared with the non-treated control (empty). However further research is necessary and ongoing in order to define the potential synergistic effect of the RGD and the PTH peptides in stimulating bone regeneration.

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