Mandibular bone repair by implantation of rhBMP-2 in a slow release carrier of polylactic acid – An experimental study in rats

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Abstract

The aim of the present study was to test the hypothesis that human recombinant bone morphogenic protein 2 (rhBMP-2) implanted in a slow release carrier of PLA can repair a non healing defect in the rat mandible and maintain the thickness of an augmented volume. P-DL-acid discs were produced and loaded with 48 µg and 96 µg rhBMP-2 and inserted into non-healing defects of the mandible of 45 wistar rats. 15 rats received implants with 96 µg rhBMP-2 (Group 2), 48 µg rhBMP-2 (Group 1) and blank implants without BMP (Group 0) each on one side of the mandible. Unfilled defects of the same size on the contralateral sides of the mandibles served as empty controls. After 6, 13 and 26 weeks implants of each group were retrieved from 5 animals each and submitted to flat panel detector computed tomography. Bone formation and thickness of augmentation was assessed by computer assisted histomorphometry. In Group 2 significantly more bone was produced than in Group 1. Implants of Group 1 induced significantly more bone than the blank controls only after 6 weeks, whereas the difference was not significant after 13 weeks and 26 weeks. Differences between Group 2 and Group 1 were clearly significant after 26 weeks. The thickness of bone tissue was maintained in Group 2 whereas it decreased in Group 1 and was negligible in Group 0. It is concluded that the PLA implants with 96 µg rhBMP-2 were able to bridge a non-healing defect in the rat mandible and maintained the thickness of an augmented volume. However, continuous supply of osteogenic signals appears to be required to compensate for adverse effects during polymer degradation.

Introduction

Repair of the facial skeleton is one of the key procedures in head and neck reconstructive surgery. The restoration of the original shape of the facial skull is a precondition for the restitution of facial appearance. Novel approaches in this field are aiming at the enhancement of bone regeneration instead of using autogenous bone grafts. One focus of this regenerative approach is the use of growth factors in skeletal repair that has been extensively researched during the past decade [1]. In particular, bone morphogenic proteins (BMPs) have been successfully applied in the reconstruction of long bones, spine and the facial skeleton in preclinical studies [2-7]. The number of materials used as carriers to accomplish delivery of BMPs at the site of implantation is huge and includes mineralized scaffolds [e.g. 8-12], metals [13], polymers [14,15], silk [16,17] and collagen [18-21]. However, most of these materials are unable to maintain a defined shape after implantation because they are either too soft or difficult to stabilize. Collagen carriers have been successfully applied in clinical studies [22,23] but very high dosages were required to induce sufficient bone formation [23]. Insufficient release characteristics to deliver the growth factor in a controlled fashion have been discussed as one of the reasons for the need to use excessive dosage in the mg range. Chemical engineering of collagen carriers and growth factors by addition of heparin binding domains has resulted in significant retardation of growth factor release and improved bone regeneration [24,25]. However, the mechanical requirements for a bone building carrier are not met by this material [26]. Other approaches have used polymers such as poly(lactide-co-glycolic acid) either as carrier alone [15], as scaffolds onto which collagen was immobilized to deliver BMPs [14] or as nano-fibrous scaffolds in combination with nanospheres containing BMPs [27].

Previous studies have shown that growth factors can also be successfully incorporated into polylactic acid (PLA) implants by gas foaming [28,29] and that BMPs included in preshaped PLA implants moulded by gas foaming were released in a retarded manner and were able to induce osteogenic differentiation in vitro [30]. These implants could thus fulfil both the mechanical requirements of providing an anatomically preformed shape and provide a controlled release characteristic of a carrier for growth factors. As a previous study had shown, that these implants were able to induce ectopic bone formation [31], it was the aim of the present study to test the hypothesis that human recombinant bone morphogenic protein 2 (rhBMP-2) implanted in a slow release carrier of PLA can repair a non healing defect in the rat mandible and maintain an augmented volume.

Materials and Methods

Implant fabrication

Gas foamed implants of 8 mm diameter and 3 mm thickness were produced as previously described (Schliephake et al. 2007a). Briefly, granular powder of amorphous poly-DL-lactic acid (Resomer R 208, inherent viscosity: 1.8 dl/g, Boehringer, Ingelheim, Germany) was mixed with aqueous solutions of rhBMP-2 at concentrations of 800 µg and 1600 µg/g polymer and subsequently lyophilized. For each implant, 0.06g of the resulting powder was filled into custom made moulds and submitted to high pressure treatment with 100 bar CO_2 for 2 hours. Implants with the lower concentration of growth factor (800 µg rhBMP-2/g polymer, Group 1) thus contained 48 µg rhBMP-2, implants with the

higher concentration contained 96 µg rhBMP-2 (Group 2). These implants have shown to release rhBMP-2 after an initial burst release for at least 24 days at a rate of < 100 ng/ml/day after day 9 in Group 1 and > 100 ng/ml/day after day 9 in Group 2 [31])

Surgery

The experiments were performed in adult male Wistar rats (weight range 330-450 g). The animals were held according to the standards of animal housing and care of the local animal research committee. The implants were inserted into non-healing full thickness defects of 5 mm diameter in the ascending ramus of the mandibles of 45 rats. They were inserted press fit into the defects after an implant of 5 mm diameter was punched out of the 8mm disc. The remaining PLA volume was minced and used to augment the lateral side of the inserted implant in order to assess the ability of the material to withstand soft tissue pressure. Blank implants (Group 0) and implants with both 48 µg (Group 1) and 96 µg rhBMP-2 (Group 2) were inserted into one side of the mandibles of 15 animals each. 5 animals from each group were evaluated after 6, 13 and 26 weeks each. Defects of the same size on the contralateral sides of the mandibles served as empty controls. At the assigned intervals, the mandibles were retrieved together with the surrounding tissue and fixated immediately in 4% buffered formalin.

The morphology of the reconstructed mandibles was assessed using flat panel detector computed tomography (fpVCT) (Performix 630, GE Medical Systems, General Electric Global Research Centers, Niskayuna, NY,USA). Focus size was 700 µm at a maximum voltage/current of 140 kV/400 mA, the flat panel detector size was 205x205 mm). Mandibles were visualised in a pseudo-3D display.

Bone formation was verified histologically from Alizarin-Methylene Blue surface stained thick sections that were fabricated from the implants after embedding into Technovit® according to Donath & Breuner [32]. The newly formed bone volume was assessed by histomorphometric analysis of cross sections through the center of the defect. The micrographic images were recorded through a video camera (Sony 3CCD; Germany) at 50X magnification, digitized and mounted together into one picture (Axiophot-System, Zeiss, Obercochem, Germany). Pixel sizes were calibrated and the area occupied by newly formed bone was assessed by pixel counting and expressed as mm^2.

The thickness of the reconstruction was assessed linearly by measuring the distance between the medial and the lateral surface of the regenerated bone at three points across the diameter of the former defect (Fig.1). The three measurements were calculated as mean value per animal and these were used to calculate mean values per group. Mean values were compared using an ANOVA with Bonferroni correction at a significance level of $p<0.05$.

Results

All animals survived and the implants healed well. No implant was lost. There was an increase in volume on all implanted sides of the mandibles and the implants appeared to be firmly fixed on palpation.

fpVCT

Empty control defects remained unfilled during the entire observation period as did the defects that had received empty PLA implants (Figs: 2a&b). Implants with 800 µg/g polymer had induced bone volume on the lateral side of the

mandibles that protruded above the cortical level between the PLA particles after 6 weeks (Fig. 2c). After 26 weeks, there was only little bone formation present above the lateral surface of the press fit inserted implant. Implants with 1600µg/g polymer had induced bone formation that was found to be grossly maintained on pseudo 3D displays from week 6 to week 26 (Fig. 2d&e).

Histology

Blank PLA implants (Group 0) did not show bone formation on the outside of the implants or between the particles used for augmentation at any interval. The implant pores were filled with connective tissue (Fig. 3a). Bone formation was visible at the contact area to the adjacent mandibular bone corresponding to the degree of implant degradation (Fig. 3b). However, no direct contact was visible between the regenerating bone and the implant surface. Empty control defects did not show substantial regeneration (Fig. 3c).

Four of the five implants with 800 µg/g polymer (Group 1) showed marked bone formation after 6 weeks that surrounded the implant body and extended between the particles on the lateral side. Haematopoietic bone marrow spaces were visible in the bone formed between the particles (Fig. 4a). After 13 weeks, bone formation extended into the resorbed cavities in one implant whereas little bone formation was found in 4 of the five implants. After 26 weeks, degradation and fragmentation was visible with very little bone formation in all implants. Multinucleated giant cells were appreciable on the surface of the implants (Fig. 4b). Empty control defects showed little bone regeneration at the defect edges.

Polymer implants with 1600 µg/g polymer (Group 2) also showed extensive formation of young woven bone after 6 weeks that covered the implant contour and extended between the particles on the lateral side of the mandible with haematopoietic bone marrow spaces in between (Fig. 5a). The newly formed

bone kept a distance of several cell layers to the implant surface. After 13 weeks, the distance between the implant surface and the adjacent bone had decreased (Fig. 5b). Spaces inside the implant created by degradation had been filled by ingrowth of newly formed bone (Fig. 5c). After 26 weeks, degradation of the implants was considerably advanced with simultaneous bone fill of the resulting cavities. Empty control defects exhibited only negligible formation of new bone tissue.

Histomorphometry

Bone regeneration extended across 1.11 mm², on average around the implants of Group 0 after 6 weeks (SD 0.97) (Fig. 6a). This did not change significantly after 13 weeks (0.99 mm² / SD 0.65) and 26 weeks (0.82 mm² / SD 0.59) (p=0.830). Empty defects showed bone regeneration of 1.18 mm² (SD 0.53) after 6 weeks, 0.12 mm² (SD 0.07) after 13 weeks and 0.22 mm² (SD 0.31) after 26 weeks. There was no significant difference between empty defects and the blank implants (6 weeks: p=0.897; 13 weeks: p=0.065; 26 weeks: p=0.283).

Bone regeneration around implants with 48 µg rhBMP-2 (Group 1) was found to have produced 4.11 mm² (SD 0.54) on average after 6 weeks. The mean value decreased to 2.15 mm² (SD 2.78) after 13 weeks and even more after 26 weeks (1.07 mm² / SD 0.62). This decrease was statistically significant (p=0.037), with the significant difference occurring between 6 and 26 weeks (p=0.042)..

Implants with 96 µg rhBMP-2 (Group 2) exhibited a mean area of 6.69 mm² bone formation (SD 2.33) after 6 weeks. After 13 and 26 weeks, the mean value had slightly decreased to 5.30 mm² (SD 1.83) and 4.36 mm² (SD 1.40), respectively. This change was not significant (p=0.180).

The difference between implants with 96 µg and the blank controls was significant after 6 weeks (p<0.001), 13 weeks (p=0.014) and after 26 weeks

($p<0.001$). Implants with 48 µg rhBMP-2 induced significantly more bone than the blank controls only after 6 weeks ($p=0.024$) whereas this difference was not significant after 13 weeks ($p=1.000$) and 26 weeks ($p=1.000$). The difference in mean values between 96 µg and 48 µg rhBMP-2 implants reached near significance after 6 weeks ($p=0.053$) and 13 weeks ($p=0.056$) but was clearly significant after 26 weeks ($p=0.014$).

Thickness of augmented area

The average distance between the medial and the lateral bone surface was 0.39 mm (SD 0.61) in the group of blank polymer implants after 6 weeks, 0.37 mm (SD 0.45) after 13 weeks and 0.40 mm (SD 0.39) after 26 weeks (Fig. 6b). This was significantly higher in the implants of Group 1 only at week six. Implants with 48 µg rhBMP-2 showed an average distance of 4.65 mm (SD 0.28); $p< 0.001$) after 6 weeks but after 13 weeks and 26 weeks this distance decreased significantly ($p< 0.001$) to 0.91 (SD 1.4) and 0.17 (SD 0.15), respectively. The thickness in the reconstructed area was maintained in the group of implants with 1600 µg/g polymer over the entire period. The distance between the lateral and medial surface of the regenerated bone after 6 weeks was 5.19 mm (SD 1.10), 4.77 (SD 0.30) after 13 weeks and 4.83 mm (SD 0.8) after 26 weeks. Differences between the three intervals were not significant ($p=0.693$).

Discussion

Mandibular reconstruction using BMPs has been evaluated in a number of experimental models with a large variety of carriers. Collagen, collagen/HA/TCP, anorganic bovine bone, hyaluronic acid, PLA/PGA coated

gelatine sponges and PGLA beads have been employed with various doses of either rhBMP-2, rhBMP4 or rhBMP7 [33-39] All carriers of these studies had been soak-loaded with their respective BMP solution, in one study rhBMP-2 was suspended in PGLA/dioxane solution and freeze-dried for implantation [40]. The variety of dosages and biomaterials as well as the lack of release data or information to calculate the final amount of BMPs applied make it difficult to compare the data with the present results, even in those studies who had used non-healing defects in the rat mandible [34-36,39]. In these studies, BMPs have proven to be of variable efficacy with respect to induced bone volume, depending on the carrier used. RhBMP-2, soak loaded into hyaluronic acid sponges had shown a smaller threshold dose (<10µg) in acute administration in these models than rhBMP4 (>10µg) [34]. These results compare well to those reported for bone induction in heterotopic sites (Uludag et al. 2000). As soak loaded carriers provide a burst release with delivery of 80-90% of activity within the first 24-48 h (Uludag et al. 2000), bone formation in these studies has been induced after a single short termed biologic impulse.

According to the in vitro data of the implants used in the present study [31], bone regeneration has occurred during a release of appr. 900 ng rhBMP-2 during the first 48 h and <100 ng/72h thereafter in Group 1 and appr. 700 ng during the first 48 h and >100 ng/72h thereafter in Group 2. The suspected amounts of BMP that were released during the first 2 days from the slow release carrier in the present study therefore will have been well below the threshold doses reported previously for soak-loaded carriers [41]. Hence, it is likely that the difference in the subsequent slow release rates between the two groups that accounted for the difference in bone formation over time showing that bone formation around implants with 48 µg rhBMP-2 occurred during early stages of implantation but decreased significantly after 13 weeks and was

reduced to the level of the unloaded implants after 26 weeks. In contrast, bone formation was maintained on a higher level across the entire observation period in the group of implants with 96µg rhBMP-2 without significant differences over time. The decrease in newly formed bone during later periods of implantation in the low dosage group may be explained by the acidic environment that occurred in the vicinity of the implants during degradation. This decrease in pH had been observed to occur in vitro after 18- 21 weeks [30]. In the higher dosage group, adequate amounts of BMPs being released from the implants could have counteracted this effect and maintained bone formation even under unfavourable conditions.

A previous study had shown that the release of rhBMP-2 from p-DL-lactic implants with 48 µg BMP has been unable to induce ectopic bone formation in the gluteal muscle of rats while those with 96 µg consistently induced osteogenesis over a period of 26 weeks [31]. The fact that bone formation occurred in orthotopic implantation during early stages around implants 48 µg in the present study may be explained by the fact that the initial burst release from the implants has been supported by the local milieu of the orthotopic site with BMPs and other factors being released from the bone surfaces. This may have allowed for induction of early bone formation but the subsequent release of rhBMP-2 has been insufficient to maintain a level of osteogenesis that is able to compensate for the adverse effects of polymer degradation.

The present study has shown that preformed gas foamed p-DL-lactic acid implants can act as a release system for recombinant human BMP2 and induce bone formation during reconstruction of the mandible. In contrast to other studies that have assessed the effect of BMP-carriers on bone formation in rat mandibles at early intervals after 4 – 8 weeks [34-36,42], also late stages of bone formation are considered in the present study. They can reflect

interference of carrier degradation with ongoing osteogenesis on the surface and the inner pores of the implants, which would have remained unrealized at commonly used intervals. It is difficult to predict the interaction between bone formation and degradation at the final stages of resorption. The morphologic findings after 26 weeks with advanced degradation suggest that the carriers in the high dosage group are gradually replaced by bone as long as an adequate level of released BMP is maintained. The metric evaluation of the thickness of the augmented mandible has shown that the PLA chips accumulated on the lateral surface of the implant had kept the thickness of the auglmented volume against the pressure of the buccal soft tissues. Also after 26 weeks, bone formation was seen on the outside of the accumulated particles. This suggests that the preservation of the augmented volume can be expected in the implants loaded with 96µg rhBMP-2.

Nevertheless, further engineering of the PLA carrier will be desirable to avoid interferences with carrier degradation. Addition of neutralizing components such as calcium carbonates and / or calcium phosphates can help to buffer the acidic degradation products occurring during resorption [43,44] and further reduce the amount of BMP required to induce bone formation that is maintained over longer periods.

Conclusions

In conclusion the present study has confirmed the hypothesis that recombinant human bone morphogenetic protein-2 can bridge critical size defects in the mandible when implanted in a slow release system of p-DL-lactic acid and that the thickness of an augmented volume can be maintained on the lateral side of the mandible. The results however also show that degradation of the p-DL-lactic acid polymer in vivo can have adverse effects on bone formation and that

maintenance of bone tissue is depending on continuous supply of osteoinductive signals.

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Legends

Fig. 1 Schematic illustration of bone thickness measurement

Fig. 2a Pseudo 3D display of a blank implant in the rat mandible with little periosteal bone formation on the augmented area after 6 weeks

Fig. 2b Pseudo 3D display of a control defect in the rat mandible with little marginal bone formation after 6 weeks

Fig. 2c Pseudo 3D display of bone formation after 6 weeks from an implant with 48 µg rhBMP-2. There is less bone volume on the outside visible compared go the high dosage group.

Fig. 2d Pseudo 3D display of bone formation after 6 weeks from an implant with 96 µg rhBMP-2

Fig. 2e Pseudo 3D display of bone formation after 26 weeks from an implant with 96 µg rhBMP-2 with gross maintenance of the augmented volume compared to 6 weeks.

Fig. 3a Micrograph showing a blank implant without bone formation and soft tissue fill of the pores, (Alizarine Methylene Blue, Magnific. 20X)

Fig. 3b Micrograph showing bone formation after 13 weeks in the vicinity of a blank implant with little newly formed bone that partially filled the space generated during degradation (Alizarine Methylene Blue, Magnific. 20X)

Fig. 3c Micrograph showing lack of bone formation after 26 weeks in a control defect (Alizarine Methylene Blue, Magnific. 20X)

Fig. 4a Micrograph showing bone formation surrounding an implant with 48 µg rhBMP-2 and filling the gaps between the polymer particles after 6 weeks (Alizarine-Methylene Blue, Magnific. 20X)

Fig. 4b Micrograph showing degradation on the surface of implants in Group 1 with the formation of multinucleated giant cells and foam cells after 26 weeks (Alizarine-Methylene Blue, Magnific. 20X)

Fig. 5a Micrograph showing formation of haematopoetic bone marrow between the augmented polymer particles from an implant with 96 µg rhBMP-2 after 6 weeks. Note the gap still present between the implant and the adjacent bone (Alizarine-Methylene Blue, Magnific. 100X).

Fig. 5b Micrograph showing approximation of bone tissue to the implant surface after 13 weeks. (Alizarine-Methylene Blue, Magnific. 100X).

Fig. 5c Micrograph showing bone formation next to a Group 2 implant after 26 weeks with bone formation in the center of the implant and with advanced degradation features (Alizarine-Methylene Blue, Magnific. 100X).

Fig. 6a Quantitative assessment of the area of bone formation

Fig. 6a Quantitative assessment of bone thickness

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