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Continuous delivery of rhBMP2 and rhVEGF165 at a certain ratio enhances bone formation in mandibular defects over the delivery of rhBMP2 alone — An experimental study in rats

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ABSTRACT

The aim of the present study was to test the hypothesis that different amounts of vascular endothelial growth factor and bone morphogenic protein differentially affect bone formation when applied for repair of non-healing defects in the rat mandible. Porous composite PDLLA/$CaCO_3$ carriers were fabricated as slow release carriers and loaded with rhBMP2 and $rhVEGF_{165}$ in 10 different dosage combinations using gas foaming with supercritical carbon dioxide. They were implanted in non-healing defects of the mandibles of 132 adult Wistar rats with additional lateral augmentation. Bone formation was assessed both radiographically (bone volume) and by histomorphometry (bone density). The use of carriers with a ratio of delivery of VEGF/BMP between 0.7 and 1.2 was significantly related to the occurrence of significant increases in radiographic bone volume and/or histologic bone density compared to the use of carriers with a ratio of delivery of ≤0.5 when all intervals and all outcome parameters were considered. Moreover, simultaneous delivery at this ratio helped to "save" rhBMP2 as both bone volume and bone density after 13 weeks were reached/surpassed using half the dosage required for rhBMP2 alone. It is concluded, that the combined delivery of $rhVEGF_{165}$ and rhBMP2 for repair of critical size mandibular defects can significantly enhance volume and density of bone formation over delivery of rhBMP2 alone. It appears from the present results that continuous simultaneous delivery of $rhVEGF_{165}$ and rhBMP2 at a ratio of approximately 1 is favourable for the enhancement of bone formation.

1. Introduction

Bone formation depends on the activity of a delicately orchestrated sequence of growth factors that coordinate angiogenesis, mesenchymal proliferation and osteogenic differentiation. A large body of research has assessed various dosages and temporal patterns of release of growth factors for the enhancement of bone repair [1–5]. Retarded delivery of either angiogenic or osteogenic growth factors has been in focus in the majority of these efforts in different experimental models [6–9]. In vivo applications of single growth factors such as bone morphogenic proteins (BMPs) have used high dosages which have been associated with considerable side effects such as implant loosening, swelling and inflammation [10–14]. In order to enhance the efficacy of growth factor delivery in bone repair and reduce the dosage of single growth factor applications, more recent strategies are directed towards the release of more than just one growth factor [15–17]. Following the idea that angiogenic stimulation of proliferation of vessels into a bone defect precedes osteogenic differentiation of undifferentiated mesenchymal perivascular cells as sources for bone formation, most of these approaches have used the combined delivery of angiogenic and osteogenic signals at various dosage levels [18–20] with a concept of an increased early delivery of angiogenic signals followed by an increased release of osteogenic growth factors. So far, the results of these approaches have been inconclusive with respect to the role of angiogenic and osteogenic stimulation in a combined release fashion [20–24]. It was therefore felt desirable to use different dosages and different ratios of vascular endothelial growth factor (VEGF) and BMP in a combined delivery approach to test the hypothesis that different amounts of VEGF and BMP differentially affect bone formation when applied for repair of non-healing defects in the rat mandible. Moreover, the hypothesis was tested that the use of VEGF can reduce the amount of BMP to induce comparable bone volumes and density. For this purpose, $rhVEGF_{165}$ and rhBMP2 were incorporated into porous composite poly-DL-lactic acid/$CaCO_3$ carriers in ten different combinations and evaluated for their ability to enhance bone formation in a mandibular defect model.

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2. Material & methods

2.1. Preparation of retarded delivery devices

Poly-DL-lactic acid/$CaCO_3$ composite granules were used for the fabrication of carriers for retarded release of growth factors. Gas foaming of this composite material in conjunction with lyophilized rhBMP2 had previously shown to produce porous carriers that provide effective retarded delivery of active rhBMP2 for 2 weeks [25]. At the same time, a drop in pH due to the occurrence of acidic degradation products during resorption that had been described before for the use of pure gas foamed poly-DL-lactide [26] was avoided by the addition of $CaCO_3$.

Sixteen grams of granular powder of amorphous poly-DL-lactic acid (PDLLA) (Resomer R 208, inherent viscosity: 1.8 dl/g, Boehringer, Ingelheim, Germany) and 4 g of spherulite-shaped precipitated calcium carbonate with the calcite crystal structure (average particle size 12 μm, Schaefer Kalk GmbH & Co. KG, Diez, Germany) were dry processed in an impact mill (NHS-0, Tokyo 143, Japan) at 5000 rpm and subsequently at 16.000 rpm [25]. The resulting granular powder (grain size: 200–400 μm) was mixed with aqueous solutions of $rhVEGF_{165}$ with 4, 25, and 100 μg/g composite (Groups 1A through 1C) and of rhBMP-2 at concentrations of 400, 800 and 1600 μg/g composite (Groups 2A through 2C). A third group received both growth factors at a lower dosage (400 μg rhBMP-2 and 25 μg $rhVEGF_{165}$, respectively) and a higher dosage (800 μg rhBMP-2 and 100 μg $rhVEGF_{165}$, respectively) in a cross over design (Groups 3A through 3D). The dosage levels of rhBMP2 were chosen based on previous experiments with pure PDLLA carriers showing that in vivo induction of bone tissue occurred at dosages of between 400μg per g polymer and the maximum applicable dosage was 1600 μg per g polymer [27,28]. The ratio of minimum dosages of rhBMP2 and $rhVEGF_{165}$ was determined from the ratio of appr. 1/100 of in vitro levels of activity (2 ng/ml for $rhVEGF_{165}$ and 300 ng/ml for rhBMP2 (data not shown)). The dosage levels for $rhVEGF_{165}$ were varied to provide a moderate (6.25 fold) and a large (25 fold) dosage increase. The dosage levels for the cross over designs were restricted to two levels per growth factor in order to limit the number of experimental groups to a feasible extent. For rhBMP2, the lower two dosages (400 μg and 800 μg) were chosen to evaluate the potential of combined delivery with rhVEGF165 to induce a level of bone formation comparable to the maximum BMP2 dosage of 1600 μg per g polymer. This maximum dosage of rhBMP2 was used as a positive control. For $rhVEGF_{165}$, the high and medium dosages (100 μg and 6 μg) were chosen to maximize possible effects of combined delivery as evaluation of delivery of VEGF alone during the course of the experiment showed that the release from medium dosage carriers approximated that of the low dosage carriers after day 3 (see below). Growth factors were purchased from ReliaTech (Braunschweig, Germany). All mixtures were subsequently lyophilized overnight.

For each carrier, 0.06 g of the growth factor loaded granular powder was filled into custom made PTFE moulds for carriers of 8 mm diameter and 3 mm thickness as described previously [26] and was submitted to supercritical carbon dioxide (CO_2 pressure: 100 bar for 2 h at 37 °C, filling time 20 min, soaking time 120 min, venting time: 20 min (5 bar/min)). The amounts of growth factors contained in the carriers of the respective groups are listed in Table 1. Blank PDLLA/$CaCO_3$-carriers served as controls.

2.2. Evaluation of growth factor release from the carriers

The carriers were evaluated in vitro for their release profile. Previous tests had shown that delivery occurred with an initial increase during the first 3 days with a subsequent gradual decline in delivery [25] which is considered to be based on superficial degradation/erosion of the polymer matrix. Blank carriers and carriers containing the a.m. amounts of rhBMP2/$rhVEGF_{165}$ were immersed into cell culture medium (DMEM, 1 g Glc, Gibco, Invitrogen, www.invitrogen.de) in BSA coated 24 well plates. The medium was collected daily. Release after 1 day, 3 days, 7 days, and weekly thereafter until the 5th week was assessed by pooling the respective aliquots. The content of rhBMP2 was assessed using a custom made commercially produced sandwich ELISA. The minimum detectable amount was 100 pg/ml rhBMP2 (Dr. Mark Hennies, Euskirchen, Germany). $rhVEGF_{165}$ was measured using a commercially available ELISA (Reliatech, Braunschweig, Germany) according to the instructions of the manufacturer. For all measurements, three carriers were evaluated at each interval. Measurements were performed twice on each carrier. In the Groups with combined growth factor loading, the ratio between released $rhVEGF_{165}$ and rhBMP2 was calculated for each time point.

2.3. Surgical procedures/implantation of carriers

The ability of the carriers to induce bone formation was assessed in a non-healing defect model in the rat mandible as described previously [27]. Briefly, carriers of 5 mm diameter were punched out of the 8 mm carrier discs. The carriers were inserted press fit into full thickness defects of 5 mm diameter in the ascending ramus of the mandibles of 120 adult male Wistar rats (weight range 330–680 g). The remaining carrier volume of the 8 mm discs was minced and used to augment the lateral side of the inserted carriers. Twelve additional animals served as controls with blank carriers being inserted on one side of the mandibles in an identical manner and empty defects on the opposite side. Each of the 10 combinations of growth factors was inserted unilaterally into the mandibular defects in 12 animals resulting in 12 carriers available for the evaluation of each growth factor dosage/dosage combination. Six carriers of each growth factor combination as well as 6 control animals were evaluated after 4 and 13 weeks each. At the end of each observation period, the mandibles were removed together with the surrounding soft tissue and fixated immediately in 4% buffered formalin.

2.4. Evaluation of bone formation

Bone formation was assessed both radiographically for evaluation of bone volume (bone area) and by histomorphometry for assessment of bone quality (bone density). For radiographic evaluation of newly formed bone, the mandibles were split in the midline and each half mandible containing a carrier was submitted to volume computed tomography (VCT) (Orange Dental). Field of view was 50 × 50 mm, focus size was 500 μm at a maximum voltage/current of 120 kV/8 mA. The maximum voxel resolution was 80 μm. Radiographic analysis was performed on axial scans perpendicular to the defect and parallel to the lower border of the mandible. One scan through the center of the defect and two scans at a distance of 1/2 the radius above and below the center were used for radiographic evaluation of each defect.

Table 1
Growth factor content of carriers.

	Blank	Group 1A	Group 1B	Group 1C	Group 2A	Group 2B	Group 2C	Group 3A	Group 3B	Group 3C	Group 3D
$rhVEGF_{165}$	0	0.24 μg	1.5 μg	6 μg	0	0	0	1.5 μg	6 μg	1.5 μg	6 μg
rhBMP2	0	0	0	0	24 μg	48 μg	96 μg	24 μg	24 μg	48 μg	48 μg