Tissue-engineered bone formation in vivo using a novel sintered polymeric microsphere matrix

We have evaluated in vivo a novel, polymer-based, matrix for tissue engineering of bone. A segmental defect of 15 mm was created in the ulna of New Zealand white rabbits to determine the regenerative properties of a porous polylactide-co-glycolide matrix alone and in combination with autogenous marrow and/or the osteoinductive protein, BMP-7. In this study four implant groups were used: 1) matrix alone; 2) matrix with autogenous marrow; 3) matrix with 20 µg of BMP-7; and 4) matrix with 20 µg of BMP-7 and autogenous marrow.

The results showed that the degree of bone formation was dependent on the properties of the graft material. The osteoconductive sintered matrix structure showed significant formation of bone at the implant-bone interface. The addition of autogenous marrow increased the penetration of new bone further into the central area of the matrix and also increased the degree of revascularisation. The osteoinductive growth factor BMP-7 induced penetration of new bone throughout the entire structure of the implant. The most effective treatment was with the combination of marrow cells and osteoinductive BMP-7.

Autogenous bone has been used extensively to repair bony defects, to stabilise fractures, and to induce fusion in the spine.1,2 The most common type of autograft is cancellous bone which is obtained from the iliac crest. The use of this type of graft gives a rate of healing of 80% to 90% and is considered to be the material of choice for bone grafting.4 Cancellous bone has specific properties related to bone regeneration including osteoconductivity, osteoinductivity, and osteogenicity.5,6

The osteoconductive property of cancellous autograft is related to the porosity of bone.7 The highly porous, scaffold-like structure of the graft allows host osteoblasts, osteoprogenitor cells and host vessels to migrate easily into the area of the defect and to begin regeneration of bone. The osteoinductive nature of cancellous autograft further aids bone regeneration by growth factors within the bone. These allow both for the differentiation and proliferation of the nearby osteoprogenitor cells and also for the revascularisation of the regeneration site, which is thought to be critical for complete healing.8-10 The osteogenic component of the autograft originates from the viable osteoblasts within the graft material. When the graft is removed from the donor site many of the osteocytes within the trabeculae die because of lack of nutrients. However, cells lining the surface of the graft survive and have been shown to produce early bone.11

The combination of these characteristics results in a graft material which is actively involved in the process of bone regeneration. The most significant limitation of autogenous grafting is the morbidity associated with the donor site. Pain, infection and inflammation are common.1

In order to avoid these disadvantages, attention has been focused on the development of a synthetic scaffold capable of mimicking the properties of cancellous autograft. In the past five years, several porous polymeric scaffolds have been produced for such a purpose.12-16 The scaffold alone serves as an osteoconductive surface for tissue regeneration.

We have developed an osteoconductive sintered microsphere matrix capable of being combined with osteogenic marrow cells and with the osteoinductive growth factor, osteogenic protein-1 (BMP-7). The matrix has a bio-mimetic structure with 100% interconnected porosity. The sintered matrix has a median pore diameter similar to the width of the internal trabeculae of trabecular bone (230 to 250 µm thickness)17 and a porosity in the range of 32% to 39%. Since the pore system of the matrix is made from biodegradable polymer, it will degrade and eventually fill with trabecular
bone, the resulting tissue will have a porosity in the range of 51% to 68%, which is similar to the 70% porosity of trabecular bone. Our aim was to evaluate the effectiveness of a novel microsphere-based scaffold in fostering bone regeneration.

**Materials and Methods**

**Production of the sintered microsphere matrix.** Microspheres were created using the solvent evaporation technique. Briefly, a 1:15 weight/volume solution of 85/15 polylactide-co-glycolide (PLAGA) (Purac molecular weight (MW) = 100 000) was created by dissolving the copolymer in methylene chloride. The copolymer solution was added drop-wise to a 1% solution of polyvinyl alcohol (PVA) (MW = 25 000, 88% mole hydrolised; Polysciences Inc, Warrington, Pennsylvania). The PLAGA/PVA emulsion was stirred at 300 rpm for ten hours. This allowed for complete evaporation of the solvent. The microspheres were isolated by vacuum filtration, washed with deionised water, air-dried for two hours and then vacuum-dried for an additional 24 hours. They were kept in a desiccator until further use. Sieves were used to isolate the microspheres into a size range of 600 to 710 µm.

The matrices were formed by pouring the microspheres into a stainless-steel mould which was heated at 160°C for four hours. The mould was allowed to cool slowly to room temperature over three hours before the samples were removed. The matrices for the in vivo implantation had dimensions of 5 x 15 mm.

**Preparation of BMP-7 matrices (in vivo implantation).** Implants were rinsed in 70% ethanol prior to UV light exposure. The sintered matrices for the in vivo study were exposed to ultraviolet light for 24 hours per side in an effort to minimise bacterial contamination. Osteoinductive matrices were prepared by incorporating 20 µg of BMP-7 onto the surface of the sintered microsphere matrices. BMP-7 (OP-1; Creative Biomolecules, Hopkinton, Massachusetts) was reconstituted in a solution of 50% ethanol/0.01% trifluoroacetic acid, resulting in a stock solution of 0.2 mg of BMP-7/ml. Using an initial test, it was determined that the matrix cylinder could hold approximately 110 µl of solution before overflowing. Under sterile conditions, the BMP-7 stock solution was added drop-wise to saturate the entire matrix. The matrices were placed into a freezer at -20°C for 20 hours after which they were freeze-dried in a lyophiliser for 24 hours. They were then kept in sealed tissue-culture plates at -20°C until required.

**In vivo ulnar nonunion segmental defect.** A segmental defect of 15 mm was created in the ulna of male New Zealand white rabbits 4 to 5 kg in weight. The experimental groups were as follows: 1) matrix alone; 2) matrix with autogenous marrow; 3) matrix with BMP-7 and 4) matrix with BMP-7 and autogenous marrow. The rabbits were allocated to the treatment groups at random with three in each group. They were anaesthetised using ketamine (50 mg/kg), xylazine (6 mg/kg) and acepromazine (1 mg/kg). The right forelimb was shaved, treated with betadine antibiotic ointment, and draped in a sterile manner. Figure 1 shows the exposed radius and ulna with application of the sintered microsphere bone matrix. Once both bones had been exposed, the segmental defect was created in the ulna with an oscillating saw using saline irrigation. For the implants containing autogenous marrow, the medullary canal of the excised bone segment was rinsed with 0.5 ml of sterile saline. The fat from the marrow was separated from the cells in the saline and removed. A single-cell suspension of marrow cells was obtained by passing cells, in saline, through an 18-gauge needle.
Radiographs of the matrix-alone group showing a) a slight radiopacity at the lower right corner of the defect (arrow) at two weeks and b) in the area of the implant at four weeks and no further signs of mineralisation in these areas at c) six and d) eight weeks.

Radiographs of the matrix-with-marrow group showing a) the formation of 4 mm of bone at the proximal end of the implant at two weeks; b) increased radiopacity with bone formation along the radius (arrow) at four weeks and progression of bone formation at c) six and d) eight weeks.
Each matrix containing marrow received 100 µl of the suspension delivered in a drop-wise manner. This method of implanting marrow cells was undertaken to mimic the procedures used in the operating room. Therefore the exact number of cells added to the matrices was not determined but the volume of cell suspension was constant for each implanted matrix. The matrices were then placed into the site of the defect so that a tight fit was obtained. The wound was closed in layers.

X-ray analysis of bone healing. At 2, 4, 6 and 8 weeks, radiographs of the forelimbs were taken to assess healing of the ulnar defect. The rabbits were sedated with intramuscular acepromazine at a dosage of 1 mg/kg. The radiographs were then scanned into a Kodak digital image-analysis camera (Kodak Co, Rochester, New York). Once the images had been imported, studies were performed to determine the optical density of the formation of new bone compared with a section of ulna without a defect.

Preparation of histological samples. At six and eight weeks, the rabbits were killed by an intraperitoneal injection of 175 mg/kg of sodium pentobarbital. The intact radius and ulna were dissected to remove any overlying tissues and placed in formalin (1% formaldehyde, Fisher Scientific, Pittsburgh, Pennsylvania) for histological preparation.

Before sectioning, samples were demineralised in 5% nitric acid for seven days. They were then embedded in paraffin and sectioned along the longitudinal axis of the ulna using a microtome. The sections (8 µm) were mounted on a slide and stained using haematoxylin and eosin. The slides were viewed using an Olympus PMG-3 light microscope (Olympus America Inc, Melville, New York) equipped with a Hitachi CCD digital camera (Hitachi America Ltd, Chula Vista, California) connected to a Dell personal computer (Dell Inc, Round Rock, Texas).

Statistical analysis. Three samples were examined for each group from which the mean and SD were determined. Student’s t-test was used to determine the differences between the means of groups. Statistical significance was set at p < 0.05. A power analysis was performed to determine the appropriate sample size. In order to recognise an increase of at least 35% in radiological densitometry between time points, as reported in the literature, with an α value of 0.05 and an SD of 10%, the sample size yielded a power of 0.89 which allowed for statistically significant results.

Results

Radiological analysis. The radiological analysis of the various preparations of the sintered matrices showed that the degree of opacity, and thus presumably the formation of bone, was dependent on the preparation of matrix implanted into the defect. The matrix-only group showed minimal bone formation. Figure 2 shows the radiograph of the matrix implanted at the site of the defect at weeks two to eight. At two weeks there were minimal signs of bone formation although a slight radiopacity was evident at the lower right corner of the defect.

The matrix-with-marrow group showed more bone ingrowth than the matrix-alone group (Fig. 3). By four
weeks there was formation of bone along the radius and at six and eight weeks this was still progressing. Radiography at six and eight weeks also indicated fusion of the ulna to the radius at the proximal end of the implant. This may have been the result of a biomechanical response to fracture or a response from the intact periosteum on the radius. This fusion aided in stabilising the end of the ulnar defect.

In the BMP-7-matrix group, there was slight formation of bone near the ends of the implant at two weeks of healing (Fig. 4). However, by four weeks, more bone was seen to be filling the entire defect, and the formation of bone was not confined to the implant. Formation of callus was also evident on this image. The radiopacity seen at this time suggested that the formation of bone had progressed along the outer surface of the matrix to the cut ends of the ulna. By six and eight weeks, the bone density had increased in the matrix. The image at eight weeks showed that the distal end of the matrix had completely fused with the ulna. It also appeared that the radius and ulna had fused.

The BMP-7-with-marrow group also showed formation of bone (Fig. 5). In comparison with the other groups, it had the greatest bone formation by two weeks. Bone regeneration had occurred throughout the length of the implant with formation of callus outside the confines of the implant area. By four weeks, more bone formation had occurred. The new bone had bridged the site of the defect linking the ends of the ulna together. The pattern of formation, however, was not as uniform as the BMP-7 matrices and near the distal end a gap was visible (Fig. 5b, arrow). It appeared that this particular implant was not placed in complete alignment with the ends of the ulna which may explain the findings. By six weeks, the size of this gap had decreased. At eight weeks the area of healing bone appeared to be similar to that at six weeks but was more radiopaque. In all the radiological images of the BMP-7-with-marrow group, there was a small area of bone formation on the outside surface of the ulna at the proximal end (Fig. 5c, arrow). After two weeks, this bony area did not change in size or radiodensity. Dissection confirmed that release of BMP-7 from the matrix had caused the mineralisation of one of the tendons of the forearm.

At two weeks the densitometric results showed an increased radiopacity which indicated formation of bone (Fig. 6a). At four weeks there was a difference in the groups when compared with a section of normal ulnar bone (Fig. 6b). There was an increased density for all groups at four weeks which was greatest in the matrix + BMP-7 + marrow group followed by the matrix + BMP-7 and then the matrix alone groups. At six weeks, the intensity of bone for all groups had increased compared with the normal ulnar bone (Fig. 6c). For all groups, the difference was less when compared to the normal segment of ulnar bone with the least being observed in the matrix + BMP-7 + marrow followed
by matrix + BMP-7 and finally the matrix-only group. By eight weeks considerable amounts of bone were observed (Fig. 6d). The optical density of the matrix + BMP-7 + marrow and the matrix + BMP-7 groups were similar to that of the segments of normal developing ulnar bone.

**Histological analysis.** The samples consisted of longitudinal sections through the implant and bone taken through the central axis of the implant. The results correlated well with the radiological images. In the matrix-only group, the samples showed formation of bone at the interface between the matrix and the ulna (Fig. 7). Bone formation was evident within the pore system of the matrix. The interior of the matrix showed invasion of fibrous tissue (Fig. 8).

The matrix-with-marrow group showed similar results to the matrix-alone group at six weeks. Figure 9 shows formation of bone in the pore system surrounding the microsphere but fibrous tissue predominated. The fibrous tissue was seen was to be vascularised but this was not the case in the matrix-alone group. Figure 10 shows fibrovascular tissue within a pore with cross-sections through several blood

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**Densitometry of radiographs after healing at a) two; b) four; c) six and d) eight weeks compared with the intact ulna.** Healing improved as BMP-7 was added to the matrix, and continued to improve when the BMP-7 was combined with marrow. By eight weeks, both the BMP-7 and BMP-7-with-marrow matrices showed radiodensity approaching that of intact bone, and significantly increased above the matrix alone. The height of the bars represents the mean optical density. The error bars indicate the SD (* p < 0.05).
vessels (arrows). The small particles seen within each vessel are red blood cells.

The BMP-7 matrix showed a substantial increase in the amount and the penetration of regenerating bone within the matrix after only six weeks. Formation of bone was more prolific and occurred directly on the surface of the microspheres and within the matrix. Figure 11 shows the formation of bone within pores located 7 mm from the distal edge. This finding was consistent with the radiography at six weeks which showed bone extending across the entire sintered matrix. At eight weeks the findings were similar with infiltration of new bone tissue into the matrix towards the centre as well as at the margins.

The BMP-7 matrix samples also showed areas of vascular tissue. This tissue was closely associated with the bone within the matrix.
This tissue organization was even more evident in the BMP-7-with-marrow group. The characteristic which set this group above the others was that the woven bone found within the matrix showed signs of remodelling into mature lamellar bone (Fig. 12).

**Discussion**

The efficacy of BMPs has been demonstrated in numerous reports describing *in vivo* studies performed on rat calvaria,21-24 rat femora,20,25,26 rabbit radii and ulnae,16,27-29 sheep vertebrae,30 orthotopic and ectopic bone sites,31,32 dog femora,33 and monkey mandible.34 Jensen et al33 inserted either a bone allograft, collagen type-I implant, hydroxyapatite (HA) implant or a collagen-coated HA implant containing BMP-7 into the femoral condyles of Labrador dogs. The addition of BMP-7 to the HA implant increased fixation of the implant by 800% and of woven bone by 400% after three weeks. Mason et al35 seeded cells which had been transfected to secrete BMP-7 onto a polyglycolic acid matrix and implanted it into an osteochondral defect of the knee in a rabbit model. After both eight and 12 weeks the BMP-7-loaded matrix had complete or nearly complete bone and regeneration of articular cartilage compared with polyglycolic acid alone. Healing of the ulnar defect was tested in primates using a BMP-7-loaded collagen carrier matrix to replace the segmental defect. After six weeks of healing, new bone was evident on radiographs and at 12 weeks histological examination showed evidence of woven and lamellar bone. By 20 weeks, advanced remodelling and revascularisation were evident.36 To date, however, no studies have compared bone tissue ingrowth *in vivo* between polylactide-co-glycolide matrices alone, matrices with BMP-7, matrices with marrow cells, and matrices with both BMP-7 and marrow cells.

The *in vivo* study revealed that the degree of bone formation was dependent on the regenerative properties of the implants. This sintered matrix by itself has been shown in prior studies to be both osteoconductive and biomechanically stable.37 In this study, it was shown that the addition of both marrow cells and an osteoinductive growth factor to the sintered matrix resulted in an optimal bone graft material with properties similar to cancellous autograft. Not only did the addition of marrow and BMP-7 increase the degree of bone formation, but it also resulted in mature bone within the relatively short period of six weeks. It is hypothesised that the seeded marrow cells differentiated directly into osteoblasts due to the presence of BMP-7. This direct conversion bypassed the cartilage precursor typically found in endochondral bone formation which resulted in faster healing. This was demonstrated by the presence of mature lamellar bone at only six weeks of regeneration.

Our study has shown that the sintered matrix holds promise as an effective bone graft material. When combined with the appropriate factors, it can aid in the regeneration of new bone. Future work will be aimed at quantifying the extent and rate of both the formation and revascularisation of bone when combined with marrow and osteoinductive factors.

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**References**


