Mesenchymal stem cells and inorganic bovine bone mineral in sinus augmentation: Comparison with augmentation by autologous bone in adult sheep

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Abstract

Our aim was to compare the osteogenic potential of mononuclear cells harvested from the iliac crest combined with bovine bone mineral (BBM) (experimental group) with that of autogenous cancellous bone alone (control group). We studied bilateral augmentations of the sinus floor in 6 adult sheep. BBM and mononuclear cells (MNC) were mixed and placed into one side and autogenous bone in the other side. Animals were killed after 8 and 16 weeks. Sites of augmentation were analysed radiographically and histologically. The mean (SD) augmentation volume was 3.0 (1.0) cm\textsuperscript{3} and 2.7 (0.3) cm\textsuperscript{3} after 8 and 16 weeks in the test group, and 2.8 (0.3) cm\textsuperscript{3} (8 weeks) and 2.8 (1.2) cm\textsuperscript{3} (16 weeks) in the control group, respectively. After 8 weeks, histomorphometric analysis showed 24 (3\%) BBM, and 19 (11\%) of newly formed bone in the test group. The control group had 20 (13\%) of newly formed bone. Specimens after 16 weeks showed 29 (12\%) of newly formed bone and 19 (3\%) BBM in the test group. The amount of newly formed bone in the control group was 16 (6\%). The results show that mononuclear cells, including mesenchymal stem cells, in combination with BBM as the biomaterial, have the potential to form bone.

Keywords: Animal trial; Biomaterial; Cell transplantation; Mesenchymal stem cells; Sinus augmentation

Introduction

Autogenous bone is the gold standard for augmentation of bone. Various methods of tissue engineering have been tested to improve osteoconductive scaffolds, but these are limited by availability and the morbidity associated with harvesting.\textsuperscript{1,2}

Usually either cells or biologically active molecules are combined with an osteoconductive scaffold.\textsuperscript{3,4} The selection of the most appropriate scaffolds is an important step towards the regeneration of hard tissue.

Bovine bone mineral (BBM) is one of the most widely used scaffolds used in sinus augmentation.\textsuperscript{5–7} Its physical properties are similar to those of human cancellous bone, both in its morphological structure and its mineral composition. To improve the rate of formation of new bone using BBM as a scaffold, several authors have studied the feasibility of combining it with growth factors such as bone morphogenetic
proteins BMP-2, BMP-7 (OP-1), or platelet-derived growth factor (PDGF). However, BMP are expensive. To form bone in rodents and higher primates, up to several mg are necessary, in contrast to the naturally occurring concentrations of BMP of 1 µg/g of bone.

The alternative idea, to combine a biomaterial with osteogenic cells, has recently been gaining interest. One option is the application of stem cells that originate from bone marrow. Mesenchymal stem cells (MSC) or bone marrow stromal cells are defined as multipotent progenitor cells with the ability to generate cartilage, bone, muscle, tendon, ligament, and fat.

Current publications in various fields have shown the benefit of using a cocktail of mononuclear cells without expanding them in vitro before reimplantation—such as grafting of autogenous marrow cells in non-union of the tibia or in peripheral arterial disease. In maxillofacial bone grafting, Smiler et al. have shown that the use of bone-marrow-derived MSC is feasible but without concentrating the cells.

We have investigated the application of mononuclear cells—among them mesenchymal and haematopoetic stem cells—together with BBM.

**Material and methods**

**Animals**

This trial was approved by the animal trial council. The mean age of the 6 sheep was 3 years (range 2.5–3.3) and mean weight 87.3 kg (range 72–98). All animals were examined, weighed, dewormed, and kept in quarantine for at least 2 weeks. Each animal was kept under the same conditions and looked after by a veterinary specialist.

**Study design**

Three animals each were assigned to an observation period of 8 and 16 weeks.

**Operation**

A bilateral sinus augmentation procedure under general anaesthesia was done through an extraoral approach. The anterior wall of the maxillary sinus was exposed inferiorly to the lower orbital rim by mobilisation of the masseter muscle and the adherent periosteum before preparation of the lateral window. The left sinus (control) was augmented with 3.5 cm² of autogenous cancellous bone harvested from the iliac crest. Bone marrow was aspirated from the iliac crest. MSC were extracted by Ficoll separation. A total of 3.5 cm² MSC and BBM (Bio-Oss®, Geistlich, Wolhusen, Switzerland) was transplanted into the right sinus (experimental). The wounds were closed with resorbable sutures.

**Collection and processing of marrow cells**

Bone marrow 60 ml was aspirated with a heparinised biopsy needle, pipetted on to Ficoll (Sigma, St Louis, MO, USA) in a ratio of 1:1, and centrifuged at 2400 rpm for 25 min. The interface layer was removed, resuspended in phosphate-buffered saline (PBS) and centrifuged again (2000 rpm for 10 min). The supernatant was pipetted off for washing. This procedure was repeated. The resulting cell pellet was resuspended in PBS. Viable nucleated cells were counted by trypan blue dye exclusion.

**Assay of colony forming units**

Cells were placed at four different densities (5000, 25,000, 50,000, 100,000 MNC/ml) in 96-well plates (Becton Dickin-son, Los Angeles, CA) and cultured in MSCBM-medium (Cambrex, USA) in a humidified atmosphere of 5% carbon dioxide at 37 °C for 7 days. The medium was initially changed after 24 h and then every second day. MSC were selected by plastic adhesion and counted under a microscope.

**Fluorescence marking**

Calcein 10 mg/kg body weight was given during the second and third weeks to the animals in the 8-week survival group and during the tenth and eleventh weeks to the animals in the 16-week survival group. Xylenol-orange 90 ml/kg of body weight was given during the sixth and seventh weeks to the animals in the 8-week survival group and during the fourteenth and fifteenth weeks to the animals of the 16-week survival group.

**Histological and histomorphometric evaluation**

After they had been fixed in formalin and dehydrated, the samples were infiltrated with resin (Heareus Kulzer, Hanau, Germany) and polymerised under ultraviolet (UV) light. Sections were cut with a diamante microsaw (Microslice, IBS, Cambridge, GB), placed on an Acrylglas carrier (Maertin, Germany) and polymerised under ultraviolet (UV) light. Eight slides of each augmentation side were selected and evaluated for each animal. The fluorescent markings were analysed before being stained with azur II and pararosanilin (Axiovert 135, Zeiss, Kochern, Germany; AnalySIS® D Soft Imaging system, Olympus Europa GmbH, Hamburg, Germany).

Results

Assay of colony forming units (CFU)

A mean of 40 million mononuclear cells (range 22–84 \( \times 10^6 \), SD 26 \( \times 10^6 \)) were obtained. A mean (SD) of 86 (50) CFU/million MNC were isolated.

Volume rendering

After 8 weeks we found volumes of 3.3 (0.9) cm\(^3\) on the experimental side and 2.8 (0.3) cm\(^3\) on the control side. After 16 weeks the corresponding volumes were 2.7 (0.3) cm\(^3\) and 2.5 (1.2) cm\(^3\).

Histological results

After 8 weeks

Connective tissue was found in all specimens from the control side. There were signs of extensive remodelling of transplanted cancellous bone and active new bony formation uniformly throughout the entire augmentation area (Figs. 1A and 2A). Osteoclasts were resorbing cancellous bone particles and neighbouring newly formed bone. Haversian systems were detected in places where there was remodelling of newly formed bone, but there was no lymphocytic infiltration.

On the experimental side there was uniform and substantial new bone formation (Figs. 1B and 2B). The bone was mature and compact with a structure containing organised osteons. There were no gaps at the bone–particle interface, and no inflammatory cells. In a few areas it was possible to find small capillaries within the augmented tissue.

After 16 weeks

On the control side the autogenous bone showed a high rate of remodelling and generally, histological findings were similar to those after 8 weeks. Histological features were also similar at 16 weeks to those at 8 weeks. Osteoclasts were in the process of resorbing BBM particles and neighbourling newly formed bone. There was no inflammatory infiltrate at the interface of the particles, and there were capillaries adjacent to the bone.

Histomorphometric results

After 8 weeks

On the control side 2.0 (0.6)% were transplanted bone, 20 (12)% newly formed bone, and 78 (9)% fibrous tissue. Of the bone 8 (6)% was formed before week 3 and 12 (5)% between weeks 3 and 8 (Fig. 3A). On the test side 24 (3)% was composed of BBM particles, 19 (11)% of newly formed bone, and 57 (9)% of soft tissue. Of the newly formed bone 3 (2)% was formed within the first 3 weeks and 15 (9)% after 8 weeks (3A).

After 16 weeks

On the control side the volume of bone graft was estimated at 4%. The rate of newly formed bone decreased to 16 (6)%, and the amount of fibrous tissue was 80 (6)%. Of the bone formed before week 11, 8 (5)% was newly formed, and a further 6 (1)% until week 16 (Fig. 3B). On the experimental
Fig. 2. Fluorescence microscopy of a histological section from an animal killed after 8 weeks. Overview of the whole augmentation area of the experimental side (BBM and bone marrow cells) (original magnification ×10). (A) The newly formed tissue is stained red and the marrow space white/blue (pararosanilin azur II staining). Because of the biomaterial there is more hard tissue than in the control group. (B) The fluorescence microscopy picture shows the formation of new bone (fluorescence marking with calcein-green and xylenol-orange).

Fig. 3. Histomorphometric analysis of the augmentation sites after (A) 8 and (B) 16 weeks for the experimental (test) and control groups. The results are displayed for each individual animal and as a mean for each group. Calcein stained areas = white; xylenol-orange = light grey; autogenous bone/BBM = grey; marrow space = dark grey.

Discussion

Grafting of the sinus floor is considered to have been successful if dental implants can be inserted, and if they are stable over time. The integration of the transplant and the osseointegration of inserted implants are faster when autogenous bone is added than with pure biomaterials. Thorwarth et al. found better bone forming kinetics in BBM with 25% autogenous bone than with BBM alone. In the present study the addition of mononuclear cells led to formation of bone similar to that of autogenous bone. Both groups showed that a mean of 19% of new bone had formed. The earlier bone formation (within the first 3 weeks in the control group) indicated a lag time for MSC. Four months after augmentation the rate of formation was similar in the two groups. The resorption rate in the control group was higher resulting in less newly formed bone and a smaller volume of transplant. The volume maintenance on the experimental side was probably caused by BBM, which does protect bone grafts from resorption. The volume of the autogenous bone decreased from the initial 3.5 cm³ to 2.5 cm³.

The yield of MNC added to BBM was not directly related to the formation of bone. For example, sheep 3 had by far the largest number of cells, but less bony regeneration than sheep 1. Other factors too could influence the rate of formation of new bone. The rate in this study was comparable to results reported elsewhere when BBM was mixed with autogenous bone, and was rather higher than that from augmentations made purely with BBM.

Our results support the data of Hernandez-Alfaro et al., who found accelerated bone formation induced by autologous bone marrow cells in a clinical feasibility study in which
they used an ex vivo cultured and autologous bone-marrow-derived cell product.\textsuperscript{23} Comparisons were made within a randomised investigation in humans who had bilateral sinus augmentation with either BBM alone or combined with so-called “tissue repair cells” (“TRC”, Astronom Biosciences, Inc.). When the radiographic images were evaluated, the bony dimensions measured had increased by 3 months postoperatively on the TRC/BBM side compared with the side given BBM alone. The histomorphometric analysis showed that the fractional surface area of BBM, as a ratio to total bone surface area, was smaller on the TRC/BBM side. A tendency to increase the formation of bone and to reduce the fractional surface area of BBM confirmed the results in the present animal trial. Bone-marrow-derived cells with BBM seem to support the formation of bone.

Some recent studies have reported a technique called “injectable bone”.\textsuperscript{24} This involves the morphogenesis of new bone tissue from isolated cells associated with bio-compatible scaffolds. One month before the operation, MSC are isolated from marrow aspirated from the patient’s iliac crest. After in vitro culture, the cells are trypsinised and used for implantation into human maxillary sinus.\textsuperscript{3,25} Despite some encouraging results, the number of patients operated on is still small and the follow up period short.

The procedures of separating, growing, and differentiating the limited number of stem cells make the clinical application difficult and expensive. More studies using control groups, and more animal or clinical trials, are necessary to evaluate the effectiveness and reliability of this method compared with the simple method of aspiration and centrifugation used in the present study.

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