T. Djapic · V. Kusec · M. Jelic · S. Vukicevic
M. Pecina

Compressed homologous cancellous bone and bone morphogenetic protein (BMP)-7 or bone marrow accelerate healing of long-bone critical defects

Accepted: 26 June 2003 / Published online: 26 August 2003
© Springer-Verlag 2003

Abstract We studied 18 adult male New Zealand rabbits with a critical right-sided ulnar defect of 15 mm. In six animals the defect was grafted with homologous compressed cancellous bone, in six animals with homologous compressed cancellous bone including 300 µg bone morphogenetic protein (BMP)-7 and in six animals with homologous compressed cancellous bone including 0.5 ml autologous bone marrow. The defect was studied using radiographs every second week for 10 weeks. At the conclusion of the experiment the animals were killed and the defect studied by histology and histomorphometry. In all animals treated with the addition of autologous bone marrow and in five of six animals treated with the addition of BMP-7, the defect healed. There was no union in animals treated with homologous compressed cancellous bone without additive. The histological picture of the regenerated area was similar in the two experimental groups. Woven bone contained small marrow spaces with fibrous tissue and capillaries. The osteoid seams were on average greater in animals that received autologous bone marrow as compared to animals that received BMP-7.

Résumé Nous avons étudié 18 lapins mâles adultes de Nouvelle Zélande avec une perte de substance ulnaire droite de 15 mm. Chez six animaux le défaut a été greffé avec de l'os spongieux compressé homologue, chez six autres avec de l'os spongieux compressé homologue in-

cluant 300 micrograms de BMP-7 et chez les six animaux restant, avec de l'os spongieux compressé homologue incluant 0,5 ml de moelle osseuse autologue. Le défaut osseux a été étudié par des radiographies toutes les 2 semaines pendant dix semaines. À la fin de l'expérience les animaux ont été sacrifiés et le défaut a été étudié par histologie et par histomorphométrie. Chez tous les animaux traités avec addition de moelle osseuse autologue et chez cinq des six animaux traités avec addition de BMP-7 le défaut a consolidé. Il n'y avait aucune consolidation chez les animaux traités avec de l'os spongieux compressé homologue sans additif. Les images histologiques de la région régénérée était semblable dans les deux groupes expérimentaux. L'os nouveau contenait des petits espaces médullaires avec tissu fibreux et capillaires. La bordure ostéοide était en moyenne plus grande chez les animaux qui avaient reçu de la moelle autologue comparés à ceux qui avaient reçu de la BMP-7.

Introduction

Surgical treatment of a skeleton characterised by bone loss offers several options that include autologous or allogenic cancellous or cortical bone, demineralised bone matrix, calcium phosphate-based bone substitutes and autologous bone marrow. Compressed cancellous bone grafts have good load-bearing properties [1, 2]. Fragments of cancellous bone harvested intraoperatively and fabricated into large grafts possess enhanced mechanical performance [2]. The choice of graft depends on the surgeon's assessment of the underlying clinical problem, i.e. osteoinductive insufficiency or structural bone loss. Numerous pre-clinical and clinical studies have provided evidence that bone morphogenetic proteins (BMPs) enable healing of critical-size long-bone defects [6, 8, 16, 21, 22, 23] and are beneficial in hip reconstructive procedures [7]. The osteogenic properties of BMP-7 have been demonstrated in rabbits and dogs [6], and BMP-2 has been successfully used to treat large bone defects and to promote spinal fusion [6, 12, 16].

T. Djapic · M. Jelic · M. Pecina (✉)
Department of Orthopaedic Surgery, School of Medicine,
University of Zagreb,
Salata 7, 10 000 Zagreb, Croatia
e-mail: marko.pecina@zg.hinet.hr
Tel.: +385-1-4819911, Fax: +385-1-4818810

V. Kusec
Clinical Institute of Laboratory Diagnosis,
Clinical Hospital Centre,
Zagreb, Croatia

M. Jelic · S. Vukicevic
Department of Anatomy, School of Medicine,
University of Zagreb,
Zagreb, Croatia

There is a long history of autologous bone marrow application both in experimental animals and humans for the purpose of osteogenic stimulation in treating delayed union and non-union of fractures and bone defects [5]. The bone-healing potential of bone marrow relies on the presence of stem cells and the ability to differentiate and renew cell population [3, 4, 5]. The stromal stem-cell system provides the basis for different tissues like bone, cartilage, muscle and other connective tissues. This potential is employed in tissue repair and regeneration, treatment of bone defects, cysts [3, 4, 5, 9] and even during distraction osteogenesis [17]. The beneficial effect of bone marrow can be enhanced by creating composite grafts with demineralised bone matrix, different carriers or stimulatory factors [11, 13, 14, 18]. However, the combination of homologous compressed cancellous bone and bone marrow has not been previously evaluated as a treatment option for large bone defects.

Crucial graft functions include scaffold for new bone ingrowths, osteoinductive proteins and cells with osteogenic potential properties [11, 20]. Finally, stable fixation is a prerogative for successful integration of the graft and subsequent bone healing. Different factors in bone-graft incorporation have been reviewed by Stevenson et al. [20].

In this study we assessed the potential of compressed homologous cancellous bone supplemented with BMP-7 or autologous bone marrow in accelerating healing of a critical long-bone defect in rabbits.

Materials and methods

Experimental animals

Eighteen adult male New Zealand rabbits were divided into three groups of six. An additional three experimental animals were sacrificed and bones used for preparation of the compressed cancellous bone cylinders. The animals were housed and all procedures performed according to the guiding principles in the Care and Use of Animals as approved by the Council of the American Physiological Society in 1991.

Surgical procedures

Morselised cancellous bone was collected under sterile conditions from the long bones and pelvis of the three experimental animals. The obtained bone material was crushed in a special device in order to produce compressed cancellous bone cylinders 4 mm in diameter.

BMP-7 was purchased from Stryker Biotech, known as "OP-1 device", (3.5 mg BMP-7 with 1 g type-I collagen) and prepared according to instructions. The final amount of BMP-7 in the graft figured 300 µg.

Under ketamine sulphate/ether anaesthesia and sterile conditions, osteotomies of the right ulna were performed with an electrically driven saw and produced a critical defect of 15 mm. In one group bone marrow (0.5 mL) was derived from the great trochanter of the right femur of each rabbit and injected into the compressed cancellous bone cylinder, which was applied as bone-defect treatment in the same rabbit. After inserting the graft the muscle and subcutaneous tissue were closed by sutures.

The following three groups were set up with different treatment regimes for the created ulnar defect:

- Homologous compressed cancellous bone, control ($n=6$)
- Homologous compressed cancellous bone and autologous bone marrow ($n=6$), and
- Homologous compressed cancellous bone and BMP-7 ($n=6$).

Radiography

Standard radiographs (distance 1 m, exposure 0.2 mAs, 40 kW) were performed immediately after surgery and at 2, 4, 6 and 8 weeks follow-up. Upon termination of the experiment at 10 weeks, the last radiographs were taken. The following scoring system was devised to evaluate bone formation during the experimental period:

- Score 1—non-union: no bridging between fragments
- Score 2—bony bridge between fragments with a width of half the ulna or less
- Score 3—bony bridge between fragments with a width of more than half but less than the entire width of the ulna
- Score 4—bone formed in the gap equal to or greater than the ulnar width.

Histology and histomorphometry

After 10 weeks the animals were killed by an anaesthetic overdose and adequate parts of the forelimbs excised. Specimens were fixed and processed undecalcified for resin embedding. The procedure comprised dehydration in acetone (70–100%) and xylene, infiltration in methylmethacrylate and embedding in a mixture of methylmethacrylate, dibutyl phthalate and benzoyl peroxide (100:25:2.5). Sections were stained with toluidine blue and viewed in visual and polarised light for identification of different tissue types and activity of bone turnover.

Histomorphometry was used for assessment of osteoid thickness in the immediate bone regenerate area. Osteocyte density was assessed in the regenerate and most distal bone area. For this purpose a camera attached to the microscope enabled transfer of the microscope image to the computer with software for measurement of areas and distances (Vamstec, Zagreb, Croatia). Osteoid thickness was measured in 2–4 equidistant sites for each osteoid seam in the regenerate area and expressed as average. Osteocyte density was performed under magnification $\times 200$ and calculated as average number of osteocytes per $100^2 \mu\text{m}$ of bone tissue on six to ten sites in the regenerate and most distant areas of bone.

Statistical analysis

Non-parametric statistical tests (Kruskal-Wallis, Wilcoxon) were used to analyse data between groups.

Results

Radiographic analysis

In the group treated with compressed cancellous bone without supplement, non-union remained throughout the experimental period. Treatment with compressed cancellous bone and autologous bone marrow showed bony healing at 10 weeks. At 4 weeks there was bony bridging of at least half the ulnar width in three of specimens and abundant callus in one of six specimens.

In the group treated with compressed cancellous bone and BMP-7, a radiodense bridging of the defect was ob-

Fig. 1A–D A compressed homologous cancellous radiodense bone cylinder without supplement fills the gap immediately after surgery (A), but very little material remains after 10 weeks (B). At ten weeks rabbits treated with compressed homologous cancellous bone with autologous bone marrow (C) or BMP-7 (D) showed complete healing with bone bridging

served in five of six rabbits by the end of the experiment. The callus was abundant in two animals. In one case absence of bone healing probably occurred due to a problem with fragment retention. A radiographic representative for each group after 10 weeks is presented in Fig. 1.

Histological analysis

Histology paralleled X-ray appearance by the end of the experimental period. In the control group the ulnar defect was 5–10 mm wide and filled with fibrous and fatty tissue, devitalised bone fragments and prolapsed muscle. Small areas of cartilage were found in some specimens. Bone remodelling was intensive in the bone tissue surrounding the defect. Dense fibrous and mesenchymal tissue located at the bone fragments indicated bone formation by intramembranous ossification in addition to the endochondral ossification (Fig. 2).

In the two groups where BMP-7 or autologous bone marrow was supplemented, a successful bone healing with bridging of the bony defect was achieved in most animals. Bone healed in all rabbits treated with autologous bone marrow and the new bone had a woven structure. Bone remodelling was intensive, with moderately wide osteoid seams in the bone-healing area. Small fragments of cartilage found in the regenerate area in some specimens indicated endochondral ossification. Relatively small bone marrow spaces in the new bone contained dense fibrous tissue, capillaries, blood vessels and lymphocytes. The combination of compressed cancellous bone and BMP-7 resulted in complete bone healing in five of six rabbits, namely those with stable fragment re-

tention. Bone remodelling and bone formation were intensive and the osteoid moderately thick in the regenerate area. The new bone had a woven structure. Devitalised bone fragments were found in the gap of some specimens. Small areas of cartilage and presence of mesenchymal tissue suggested that ossification was both endochondral and intramembranous. Bone marrow spaces were relatively small in the bone regenerate area and filled with dense fibrous tissue and capillaries. Osteoid thickness in the regenerate area was significantly different between the three groups ($P < 0.03$), being the greatest in rabbits treated with the compressed cancellous bone and autologous bone marrow.

Osteocyte density was on average statistically greater in the regenerate area than in the distal area ($P < 0.01$) but did not differ between groups either for the regenerate or distal bone (data not shown). This was indicative of intensive bone formation process in this area.

Discussion

Successful bone healing of the critical ulnar defect was observed by the end of the 10-week experimental period in most specimens treated with autologous bone marrow or with BMP-7-supplemented compressed homologous cancellous bone. Application of compressed cancellous bone alone was not effective in this experimental model. The study provided evidence that addition of bone marrow to the compressed cancellous bone scaffold promoted osteogenesis and generated bone healing of the critical long-bone defect.

It should be stressed that this grafting combination has not been previously used in the treatment of large bone defects reported to produce successful outcome. Osteoinductive potential of the bone marrow was observed radiographically as early as 4 weeks after surgery. In 50% of the experimental animals, the bony bridge was already half of the ulnar width at this time point. Stability of the ulnar fragments was an additional favourable factor contributing to the successful treatment outcome.

Fig. 2 A Specimen from rabbit treated with compressed cancellous bone without supplement. The gap was filled with fibrous and adipose tissue, prolapsed muscle and pieces of devitalised bone (DB). Bone fragments (BF) were undergoing intensive remodelling ($\times 40$, toluidine blue). (B) Specimen from rabbit treated with compressed cancellous bone and bone marrow. New bone (NB) formed in the ulnar defect contained bone marrow spaces (BMS) with dense fibrous tissue and capillaries ($\times 40$, toluidine blue). (C) Specimen from rabbit treated with compressed cancellous bone and BMP-7. Bone marrow spaces (BMS) were filled with fibrous tissue in the new bone (NB) formed in the defect in ($40\times$, toluidine blue). (D) Thickness of the osteoid seams in the regenerate area differed between the treatment groups ($P=0.03$). 2: Control group (homologous compressed cancellous bone). 3: Group treated with homologous compressed cancellous bone and autologous bone marrow. 4: Group treated with homologous compressed cancellous bone and BMP-7

Fresh bone marrow as a bone grafting agent has been thoroughly studied in both experimental animals and humans [5]. It has been shown that autologous cultured bone-marrow-derived mesenchymal stem cells on porous ceramic cylinders promoted healing of critical-sized segmental bone defects in dogs [4]. It was stated that the osteogenic potential of cultured mesenchymal stem cells was superior to fresh bone marrow by promoting faster and more extensive new bone formation [4].

Similar outcome was observed when BMP-7 was supplemented to compressed homologous cancellous bone. Filling of the bone defect and an abundant callus formed in five of six specimens. Failing in one case was caused by fragment instability. It is well established that BMP-7 is efficient in healing of critical-size long-bone defects in rabbits and dogs, as well as stimulating new bone formation around cementless implants grafted with bone or a bone substitute [6, 7, 15]. In an earlier study BMP-7 was used in a rabbit ulnar non-union model [6]. Demineralised bone matrix served as carrier for BMP. All implants induced a complete radiographic osseous union within eight weeks. The authors also point to the advantages of the use of highly purified recombinant forms of osteogenic proteins as compared to purified BMP preparations, thereby eliminating the risk of disease transmission and avoidance of immunogenic problems [6]. Salkeld et al. reported that ulnar critical-size defects in dogs treated with BMP-7, independent of the amount used, obtained greater mechanical strength at twelve weeks than that obtained by autografted bone alone [19]. Histological evidence of osteogenesis in mandibular reconstruction in patients was demonstrated for naturally derived BMPs extracted from bovine bone [10]. In a canine model of critical-sized defect, allograft bone combined with the osteogenic protein 1 (OP-1) device was used,

resulting in optimal healing [19]. In a similar experiment (canine ulnar defect) complete bridging was observed radiographically at eight weeks [6]. Choice of an ideal BMP delivery system still remains a challenge. It has been emphasised that a carrier is necessary to prevent rapid diffusion of BMP and to create a zone of its high concentration and slow release [6, 8].

We observed in this study that the course of healing and histological appearance in groups treated by compressed homologous cancellous bone with either autologous bone marrow or BMP-7 were comparable. Histological analysis of the specimens performed at the end of experiment showed similar findings for groups treated by compressed homologous cancellous bone combined with either autologous bone marrow or BMP-7. Evidence of both endochondral and intramembranous ossification was found in both groups. The regenerate bone found in the initial defect was woven and showed greater osteocyte density than bone in distal areas, as a consequence of intensive healing processes. Osteoid seams were greater in the group treated by compressed homologous cancellous bone with autologous bone marrow than in the group with BMP-7. This finding could indicate differences in cell activity between these two experimental groups.

Autologous cancellous bone graft is considered a gold standard against which all other bone graft substitutes are judged [11, 20]. Bone regeneration in this type of bone graft relies on viable osteoblasts and osteoprogenitor cells [20]. Homologous cancellous bone grafting is less effective despite a lack of viable osteoblasts and osteoprogenitor cells. Application of the compressed homologous cancellous bone in our study did not result in bone healing.

This study showed that BMP-7 has a potential to stimulate osteogenesis in a comparable fashion to autologous bone marrow. Clinical use of autologous bone marrow for assisting bone healing is hindered by the restricted amount of bone marrow available and morbidity related to the site of bone marrow aspiration [5].

References

1. Bilic R, Korzinek K (1987) Results of scaphoid non-union treatment by the Matti-Russe procedure using compressed cancellous bone. *Unfallchirurg* 90:134–138
2. Bonutti PM, Cremens MJ, Miller BG (1998) Formation of structural grafts from cancellous bone fragments. *Am J Orthop* 27:499–502
3. Bruder SP, Kraus KH, Goldberg VM, Kadiyala S (1998) The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. *J Bone Joint Surg [Am]* 80:985–996
4. Caplan AI, Bruder SP (2001) Mesenchymal stem cells. Building blocks for molecular medicine in the 21st century. *Trends Mol Med* 7:259–264
5. Connolly JF (1995) Injectable bone marrow preparations to stimulate osteogenic repair. *Clin Orthop* 313:8–18
6. Cook DS, Rueger DC (2002) Preclinical models of recombinant BMP induced healing of orthopaedic defects. In: Vukicevic S, Sampath KT, eds. *Bone morphogenetic proteins*. Birkhauser Verlag, Basel 121–144
7. Cook SD, Barrack RL, Shimmin A, Morgan D, Carvajal JP (2001) The use of osteogenic protein-1 in reconstructive surgery of the hip. *J Arthroplasty* 16:88–94
8. Croteau S, Rauch F, Silvestri A, Hamdy RC (1999) Bone morphogenetic proteins in orthopedics: From basic science to clinical practice. *Orthopedics* 22:686–695
9. Delloye C, Docquier PL, Cornu O, Poilvache P, Peteres M, Woitrin B, Rombouts JJ, De Nayer P (1998) Simple bone cysts treated with aspiration and single bone marrow injection. A preliminary report. *Int Orthop* 22:134–138
10. Ferretti C, Ripamonti U (2002) Human segmental mandibular defects treated with naturally derived bone morphogenetic proteins. *J Craniofac Surg* 13:434–444
11. Finkemeier CG (2002) Bone-grafting and bone-graft substitutes. *J Bone Joint Surgery [Am]* 84:454–464
12. Fischgrund JS, James SB, Chabot MC, Hankin R, Herkowitz HN, Wozney JM, Shirkhoda A (1997) Augmentation of autograft using rhBMP-2 and different carrier media in the canine spinal fusion model. *J Spinal Disord* 10:467–472
13. Gazdag AR, Lane JM, Glaser D, Forster RA (1995) Alternatives to autogenous bone graft: Efficacy and indications. *J Am Acad Orthop Surg* 3:1–8
14. Goshima J, Goldberg VM, Caplan AI (1991) The origin of bone formed in composite grafts of porous calcium phosphate ceramic loaded with marrow cells. *Clin Orthop* 269:274–283
15. Jensen TB, Overgaard S, Lind M, Rahbek O, Bunger C, Soballe K (2002) Osteogenic protein 1 device increases bone formation and bone graft resorption around cementless implants. *Acta Orthop Scand* 73:31–39
16. Pecina M, Giltaij LR, Vukicevic S (2001) Orthopaedic applications of osteogenic protein-1 (BMP-7). *Int Orthop* 25:203–208
17. Richards M, Huibregtse BA, Caplan AI, Goulet JA, Goldstein SA (1999) Marrow-derived progenitor cell injections enhance new bone formation during distraction. *J Orthop Res* 17:900–908
18. Salama R, Weissman SL (1978) The clinical use of combined xenografts of bone and autologous red marrow. A preliminary report. *J Bone Joint Surg [Br]* 60:111–115
19. Salkeld SL, Patron LP, Barrack RL, Cook SD (2001) The effect of osteogenic protein-1 on the healing of segmental bone defects treated with autograft or allograft bone. *J Bone Joint Surg [Am]* 83:803–816
20. Stevenson S, Emery SE, Goldberg VM (1996) Factors affecting bone graft incorporation. *Clin Orthop* 324:66–74
21. Tagil M, Jeppsson C, Aspenberg P (2000) Bone grafting incorporation. Effects of osteogenic protein-1 and impaction. *Clin Orthop* 371:240–245
22. Vukicevic S, Stavljenic A, Pecina M (1995) Discovery and clinical application of bone morphogenetic proteins. *Eur J Clin Chem Clin Biochem* 33:661–671
23. Zabka AG, Pluhar GE, Edwards RB 3rd, Manley PA, Hayashi K, Heiner JP, Kalscheur VL, Seeherman HJ (2001) Histomorphometric description of allograft bone remodeling and union in a canine segmental femoral defect model. A comparison of rhBMP-2, cancellous bone graft, and absorbable collagen sponge. *J Orthop Res* 19:318–327