The treatment of osteomyelitis with gentamicin-reconstituted bone xenograft-composite

Xiang-dong Li, Yun-yu Hu
From the Fourth Military Medical University, Xi’an, China

We have developed a new drug-delivery system using reconstituted bone xenograft to treat chronic osteomyelitis. This material, which has the capabilities of osteoinduction and osteoconduction, was supplemented with up to 2000 times the minimum inhibitory concentration of gentamicin against Staphylococcus aureus to prepare a gentamicin-reconstituted bone xenograft-composite (G-RBX-C). In a rabbit model, we evaluated the release of gentamicin from this composite in vivo, its capability for induction of ectopic bone and the repair of segmental defects of the radius.

There was a high level of concentration of antibiotics, which was sustained for at least ten days. In the study of induction of ectopic bone, there was abundant woven bone in the G-RBX-C group two weeks after operation. At 16 weeks after implantation of G-RBX-C the radial defects had been repaired, with the formation of lamellar bone and recanalisation of the marrow cavity. Our findings suggest that G-RBX-C may be useful in the treatment of chronic osteomyelitis.

Materials and Methods

Preparation of bone morphogenetic protein (BMP). We used calf cortical bone to obtain BMP according to the method described by Urist et al., except that our end product was a crude extract of BMP. We then implanted 2 mg and 8 mg of the product separately into the left and right thigh muscle pouches of mice for bioassay. Histological and radiological studies confirmed that the extracted materials had efficient osteoinductive ability (Fig. 1) and the more BMP which was implanted the greater was the formation of new bone. Electrophoresis in SDS-PAGE showed that it had the same pattern as that already reported.6,7

Electrodes were inserted into bone and the bone was then excised. The bone was then placed into a 37°C water bath and maintained at this temperature for 15 minutes. The bone was then transplanted into the left and right thigh muscle pouches of mice. Histological and radiological studies confirmed that the extracted materials had efficient osteoinductive ability (Fig. 1) and the more BMP which was implanted the greater was the formation of new bone. Electrophoresis in SDS-PAGE showed that it had the same pattern as that already reported.6,7

The operative treatment of chronic osteomyelitis may produce large bone defects which should be filled to reduce recurrence. Various antibiotic carrier systems have been developed, particularly antibiotic-impregnated polymethylmethacrylate (PMMA) beads which are widely used,1,2 but require removal at a subsequent operation. Recently, drug-delivery systems using resorbable materials such as collagen,3 fibrinogen4 and polylactic acid5 have been developed which do not require removal, but do not replace bone grafting.

X. D. Li, MD, Lecturer
Y. Y. Hu, MD, Professor of Orthopaedic Surgery
Institute of Orthopaedics, Xi-Jing Hospital, Fourth Military Medical University, Xi’an 710032, People’s Republic of China.

Correspondence should be sent to Dr X. D. Li.

©2001 British Editorial Society of Bone and Joint Surgery 0301-620X/01/711271 $2.00
Treatment of calf cancellous bone. Granules of calf cancellous bone, 5 × 5 × 5 mm in size, were washed with distilled water, defatted using chloroform and ethanol, and then soaked in hydrogen peroxide for deproteinisation. After washing with cold water, the bone granules were partially decalcified by immersing them in 0.6N HCl for three minutes at 25°C, and then freeze-dried. The bone granules were then soaked in hydrogen peroxide for deproteinisation. After washing with cold water, the bone granules were partially decalcified by immersing them in 0.6N HCl for three minutes at 25°C, and then freeze-dried. The bone granules were then soaked in hydrogen peroxide for deproteinisation. After washing with cold water, the bone granules were partially decalcified by immersing them in 0.6N HCl for three minutes at 25°C, and then freeze-dried. The bone granules were then soaked in hydrogen peroxide for deproteinisation. After washing with cold water, the bone granules were partially decalcified by immersing them in 0.6N HCl for three minutes at 25°C, and then freeze-dried. The bone granules were then soaked in hydrogen peroxide for deproteinisation. After washing with cold water, the bone granules were partially decalcified by immersing them in 0.6N HCl for three minutes at 25°C, and then freeze-dried. The bone granules were then soaked in hydrogen peroxide for deproteinisation. After washing with cold water, the bone granules were partially decalcified by immersing them in 0.6N HCl for three minutes at 25°C, and then freeze-dried. The bone granules were then soaked in hydrogen peroxide for deproteinisation. After washing with cold water, the bone granules were partially decalcified by immersing them in 0.6N HCl for three minutes at 25°C, and then freeze-dried. The bone granules were then soaked in hydrogen peroxide for deproteinisation. After washing with cold water, the bone granules were partially decalcified by immersing them in 0.6N HCl for three minutes at 25°C, and then freeze-dried.

SEM of the cancellous bone showed a regular porous structure, with a pore size of 300 to 500 μm in diameter and communicating pores of 50 to 80 μm. The thickness of the wall between the pores was 60 to 100 μm (Fig. 2A).

Recombination of bBMP and the calf cancellous frameworks. We redissolved 800 mg of bovine BMP (bBMP) aggregates in 4M guanidine hydrochloride, added 4 g of partially decalcified cancellous bone and removed the air from the pores of the cancellous bone under vacuum. The resulting composite, which was designated as reconstituted bone xenograft (RBX), was dialysed against distilled water, freeze-dried and sterilised with ethylene oxide. SEM of the cancellous bone showed a regular porous structure, with a pore size of 300 to 500 μm in diameter and communicating pores of 50 to 80 μm. The thickness of the wall between the pores was 60 to 100 μm (Fig. 2A).

Preparation of gentamicin-reconstituted bone xenograft-composite. The reconstituted bone xenograft was impregnated in 2 mg of gentamicin solution for 24 hours, then freeze-dried. The resulting granule was covered with a mixture of 2 mg of gentamicin and gelatin, freeze-dried and sterilised by gamma irradiation (1.5 Mrads) for eight hours.

Bioassay of gentamicin-reconstituted bone xenograft-composite. We used 36 mice weighing 20 to 22 g randomly divided into three groups. Their right femoral muscle pouches were implanted with G-RBX-C, RBX and calf cancellous framework, respectively. Six animals were killed two weeks after operation. The samples were harvested and one half in each group was demineralised with formic acid and stained with haematoxylin and eosin. After staining, each section was examined histologically by light microscopy. The other half was homogenised in 0.5 ml of phosphate buffer at pH 7.9. The supernatant fluid was centrifuged off and the activity of alkaline phosphatase (ALP) was measured.

Release of gentamicin from G-RBX-C in vivo. We used 36 male New Zealand White rabbits weighing 2.2 to 3.0 kg. Under general anaesthesia we created an osteoperiosteal defect of 15 mm through the whole thickness of the shaft of both radii by removing the segmental bone. In one half of the rabbits, the defects in the left radii were implanted with three G-RBX-C granules which contained 12 mg of gentamicin and 6 mg of bBMP with a BMP-to-carrier ratio of 1:5, and those on the right were implanted with three RBX granules. In the other half, the defects were left untreated as blank controls. The approximate muscles held the graft in place and no internal fixation or external splints were necessary. Blood samples were taken from the ear vein of the G-RBX-C-implanted rabbits at 12 hours and on days 1, 2, 3, 5, 7 and 10 for determination of the level of gentamicin in serum. After coagulation and centrifugation, the serum was stored at -20°C until assay. The animals were killed at 4, 8 and 16 weeks for radiological and histological evaluation.

Microbiological determination of gentamicin. The concentrations in the serum and tissue were measured by a disc-diffusion assay, using Bacillus subtilis ATCC 25923 as the test bacterium. This particular strain of staphylococcus has a minimum inhibitory concentration (MIC) to gentamicin of 2 μg/ml.8

Statistical analysis. We performed statistical analysis using Student’s t-test. The data are presented as the mean ± SD. Statistical significance was set at p < 0.05.

Results

Release of gentamicin from G-RBX-C in vivo. The results showed that in the muscle tissue around the G-RBX-C, the concentration of gentamicin was as high as 144.5 μg/ml at 12 hours after implantation and remained above the MIC of Staphylococcus aureus (< 2 μg/ml) for ten days (Fig. 3).

The serum gentamicin concentration reached its peak level (3.0 μg/ml) 12 hours after implantation of G-RBX-C in rabbits and then rapidly decreased. The serum levels of gentamicin in all rabbits were considerably below the toxic range (> 10 μg/ml) (Fig. 4).
Bioassay of G-RBX-C. In the G-RBX-C and RBX animals, islands of cartilage were seen with ossification occurring in the centre, and foci of chondroid, osteoid, and woven bone were observed in the pores of grafts or between the grafts and the surrounding muscle (Figs 5a and 5b). In the control implants of carrier alone, richer granular tissue was noted and no visible osteochondral differentiation was found. More than half of the cancellous bone was resorbed and degraded with a fibrous cast surrounding the remnants (Fig. 5c).

Activity of ALP in the G-RBX-C and RBX animals was significantly higher than that in the calf cancellous framework (p < 0.01) (Fig. 6).

Repair of osseous defects with G-RBX-C in the rabbit model. On day 3, spindle-shaped masses were seen against a background of slight swelling centred over the site of implantation, but there was no redness of the skin, and no exudation. The masses dwindled in size and increased in consistency in one week and the wound healed without incident. On the control side, no conspicuous mass was seen and the wound healed by first intention.
Radiological findings. In the radii implanted with G-RBX-C and RBX, considerable callus was seen which was dense and irregular in shape with the implant itself largely resorbed at four weeks. At eight weeks, the defects were filled with callus, and conspicuous remodelling was seen in some of the specimens. At 16 weeks, all the defects implanted with G-RBX-C and RBX had been largely repaired with formation of laminar bone and recanalisation of the marrow cavity (Fig. 7a), whereas nonunion of the defects occurred in the control radii (Fig. 7b).

Histological findings. Four weeks after operation, at the sites of implantation of G-RBX-C and RBX, marked chondrogenesis and osteogenesis were seen with ingrowth of tissue into the graft pores and formation of new bone, but no indications of inflammatory infiltration and phagocytosis. At eight weeks, increased amounts of new bone tissue were found within and around the graft pores. There was pronounced creeping substitution in some areas with the graft disorganised, largely resorbed and combined with the new bone. The periosteum, lamellar bone and recanalisation of the marrow cavity were identified in most of the specimens at 16 weeks after implantation, and the defects were found to have repaired to an apparent completeness by that time (Figs 8a and 8b). In the control group bony defects were filled with scar tissue (Fig. 8c).

Discussion

Chronic osteomyelitis is clinically refractory. Surgical debridement of necrotic tissue and administration of antibiotics are the principal methods of treatment. Because antibacterial levels of antibiotics cannot be delivered to infected bone intravenously without producing systemic toxic effects, local administration such as closed irrigation and suction,10 local injection,11 regional perfusion of a limb,12 and implantable antibiotic pumps13 have been used but are clinically inconvenient. Antibiotic-loaded PMMA beads, the only drug-delivery system which has been used successfully,14 provide a simple method, but require a second operation for removal because they are not degradable in vivo. Attention has therefore been focused on developing alternative absorbable vehicles for drugs which also provide high, effective concentrations of antibiotics at the site of infection with no systemic effects.15 To date, several antibiotic vehicles have been clinically and experimentally tested including fibrin-antibiotic combinations, autologous blood clot, tri-calcium-phosphate ceramic, protamine-antibiotic mixture, collagen-antibiotic floss and others.16 Although these materials can be absorbed in the body, the defects still require bone grafting. Development of a grafting material which also provides a high, effective concentration of antibiotics at the site of the wound with no systemic effects, may have potential for use in the treatment of chronic osteomyelitis.

We have reported the use of a new grafting material, reconstituted bone xenograft, based on studies of the antigenicity of the xenograft.17-19 By using frozen sections and immunofluorescence assay on undecalcified specimens, we found that the antigenicity of the graft was primarily located in the osteocytes and endothelium of the Haversian canal, with very little in the collagen matrix.17 Because the antigenicity and inductivity of the xenograft share a common protein base none of the traditional methods for the treatment of xenografts, such as freezing, freeze-drying, decalciifying, boiling, radiation and deproteinisation are satisfactory. Some are too weak to eliminate the anti-
genicity in the xenografts, while others are so strong that they destroy all the bioactive factors. This suggests that the key to the solution lies in treating the two separately. By chemical treatment of the calf cancellous bone to make it a carrier free from antigenicity, and extraction of BMP, a highly osteoconductive material, from calf cortical bone, followed by recombination of the antigen-free framework with BMP, we have developed a new grafting material, reconstituted bone xenograft, which has strong osteoinductive powers without evoking immune rejection.

As a material for repair of bone defects, BMP depends on a suitable framework for mechanical support and release. Although many biomaterials have been studied, such as hydroxyapatite (HA), β-tricalcium phosphate (β-TCP), polylactide and polyglycolide (PLA/PGA) and plaster of Paris (POP), most are difficult to absorb and remain in the body for a long time, possibly several years. By contrast, the treated cancellous bone used in our study has several advantages. It has a natural porous structure with varying pore sizes at different sites, which our studies and those of Flatley, Lynch and Benson have shown to be suitable for ingrowth of tissue. It is easy to resorb not only because of its porosity but also because of the homogeneity of bone structures in vertebrate species, which allows host cells to gain easy access to the graft materials, just as in the remodelling process. Biomaterials are superior to others in that they carry with them information which enhances the attachment and differentiation of the cells. The collagen matrix contained in the cancellous bone provides BMP with an optimal combination with and release from the framework, giving full scope to the role of BMP as an osteoinductive agent, which compares very favourably with most artificial materials. Because of the ready availability of animal tissue, this type of graft may be economical, convenient and unlimited.

Gentamicin has broad-spectrum antimicrobial activity against Gram-negative and Gram-positive organisms. Most of the major causative bacteria of chronic osteomyelitis are sensitive to gentamicin. It does not provoke an allergic reaction, and has good penetrating ability in bone tissue. It has been suggested that RBX combined with a high concentration of antibiotics will reduce the recurrence of infection and stimulate osteogenesis, and can be used as an effective method in the treatment of chronic osteomyelitis. Multiple operations would be avoided.

We impregnated RBX with 2 mg of gentamicin solution and then covered it with a mixture of 2 mg of gentamicin.
and gelatin. The pharmacokinetics of the composite in vivo showed that therapeutic concentrations of antibiotic remained at the site of implantation for ten days, which was sufficient to provide antimicrobial activity.

The serum concentration of gentamicin, which is important for evaluating systemic side-effects, reached its peak level (3.0 \(\mu g/ml\)) at 12 hours after implantation of the G-RBX-C in rabbits and then rapidly decreased. This was well below the toxic range (> 10 \(\mu g/ml\)).

Evaluation of the capability of G-RBX-C for formation of ectopic bone showed that there was abundant woven bone in both the G-RBX-C and RBX groups two weeks after implantation. Analysis of the data indicated that the differences in the activity of ALP between the G-RBX-C and RBX groups was not statistically significant (\(p > 0.05\)). This suggests that the high local concentration of gentamicin does not inhibit the formation of ectopic bone of G-RBX-C.

The rabbit model for sequential defects of the radius was designed to allow for assessment of the incorporation of G-RBX-C in vivo in a weight-bearing limb devoid of fixation which might alter the healing process. In both the G-RBX-C and RBX sides, chondral bone and islands of new bone were seen at the site of defects four weeks after operation. A number of new ossicles had formed which increased in size by the 8th week and by the 16th week an early medullary canal appeared to be forming which contained substances resembling elements of marrow. There was no difference radiologically and histologically in healing between the G-RBX-C and RBX groups. The high local concentration of gentamicin does not alter the healing process which occurs with RBX alone.

These observations, together with the release profile in vivo, suggest that G-RBX-C may be a useful method for the clinical treatment of chronic osteomyelitis. We are currently studying its effectiveness in the treatment of chronic osteomyelitis in an animal model.

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

References