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EXPERIMENTAL STUDIES ON BONE INDUCING ACTIVITY OF COMPOSITES OF ATELOPEPTIDE TYPE I COLLAGEN AS A CARRIER FOR ECTOPIC OSTEOINDUCTION BY rhBMP-2

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SUMMARY : Atelopeptide type I collagen derived from fresh porcine skin was evaluated as a carrier for ectopic osteoinduction by recombinant human bone morphogenetic protein-2(rhBMP-2) in rats. Four treatment groups(N=5) were examined: a control group in which only atelopeptide type I collagen was implanted, and groups II, III and IV in which atelopeptide type I collagen with 2,10,50μg of rhBMP-2 was implanted in to the calf muscles of 10-week-old Wistar rats, respectively. Four weeks after the implantation, soft X-ray and light microscopic examinations were performed. In addition, calcium(Ca) content and alkaline phosphatase(ALP) activity were evaluated. New bone formation in the implanted regions(groups II, III and IV) were revealed. Bone formation was induced in the implanted region of even 2μg of rhBMP-2(group II), and its degree was dependent on the dose of rhBMP 2. These results indicate that atelopeptide type I collagen is an effective carrier for ectopic osteoinduction by rhBMP-2 and may act as a carrier for rhBMP in reconstructive surgery for bony defect and augmentation.

Wang et al. succeeded in purifying BMP which has a molecular weight of 30KDa under nonreduction conditions, and 30, 18 and 16KDa under reduction conditions (1). Wozney et al. succeeded in cloning the cDNA of BMP-1,2,3,4 by a genetic technology method (2), and then the cloning of cDNA BMP-5,6,7 were succeeded (3-6). On the other hand, because of rapid diffusion of BMP when implanted in vivo, a slow delivery system as a carrier for BMP is demanded. Recent animal experiments used inactive bone matrix, 4M guanidine-HCl

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extracted residual of demineralized bone matrix of animals as a carrier for rhBMP (7-10). In the near future, purier or artificial materials will be required to act as substitute carriers for rhBMP. These materials should be mass-producible, have low antigenicity, high biocompatibility and biodegradability, and not inhibit bone induction. Until now there was no report on the use of atelopeptid type I collagen as a carrier for rhBMP-2. In this study, atelopeptid type I collagen derived from porcine skin was used as a carrier for rhBMP-2. This study was conducted with the following three intentions; first, whether or not rhBMP-2 with type I collagen as a carrier indicates osteoinducing activity in rats; second, whether or not the Ca content and the ALP activity increase are dependent on the dosage of rhBMP-2; third, whether or not atelopeptid type I collagen used as a carrier shows low antigenicity.

MATERIALS AND METHODS

Animals: Twenty male 10 week-old Wister rats, weighing 230-260g, were used. They were fed rodent chow (Certified diet MF; Oriental Koubo Inc. Tokyo, Japan) pre-postoperatively.

Implant materials: The rhBMP-2 was provided by the Genetics Institute, Cambridge, Massachusetts through Yamanouchi (Yamanouchi Inc. Tokyo, Japan). Atelopeptide type I collagen solution (3mg/ml, pH3.0. Cellmatrix LA; Nitta Gelatin Inc. Osaka, Japan) was used as the carrier for rhBMP-2 in this study. This material was a highly purified type I collagen derived from fresh porcine skin via proteolytic enzyme treatment under particular conditions. A total of 310μg of rhBMP-2 was divided into groups (group II, III, IV, each of 2, 10 and 50μg of rhBMP-2, N=5), and dissolved in 1ml of atelopeptide type I collagen solution. And then lyophilized (EYELA FDU-830; Tokyo Rikakikai Inc. Tokyo, Japan) and formed the same discal shape (4mm in diameter, 1.5mm in thickness). Whereas in the control group (N=5), only atelopeptide type I collagen solution without rhBMP-2 was lyophilized and formed the discal shape and was then used .

Operative procedure: All rats were anesthetized with intraperitoneal administration sodium pentobarbital (5.0mg per 100g of body weight). Following disinfection of the operative region, lyophilized discal specimens were implanted discreetly in the right calf muscles.

Analysis: Radiographic and Quantitative evaluation; Four weeks after the implantation, the implanted region were excised including the surrouding tissue, and soft X-rayed (SOFRON; SRO-M50, Sofron Inc. Tokyo, Japan), weighed and then homogenized in 0.25M sucrose in a Polytron homogenizer (Bio-Mixer; typeABM, Nissei Inc. Osaka, JAPAN). The sediment was demineralized in 0.5N HCl, and the Ca content of the soluble fraction was determined by the orthocresolphthalein complexone method(11). The ALP activity level and total protein in the resultant supernatant were determined by the 4NPP method (12), respectively. The Ca content (μg)/mg of tissue and the ALP activity (IU)/mg of protein were used as indices for bone formation.

Histologic analysis: The specimens submitted for histologic analysis were fixed in phosphate-buffered formaldehyde and demineralized in EDTA and embedded in paraffin. Four-micrometer sections were cut and stained with hematoxylin and eosine.

RESULTS

Radiographic evaluation: Soft X-ray revealed opaque shadow morphologically identical to the implanted specimens (Fig1). This opaque shadow was observed in each of the specimens that contained 2, 10, and 50μg of rhBMP-2. In the control group, however, such radiopaque images were not evident. This opaque shadow increased almost in proportion to the amount of rhBMP-2.

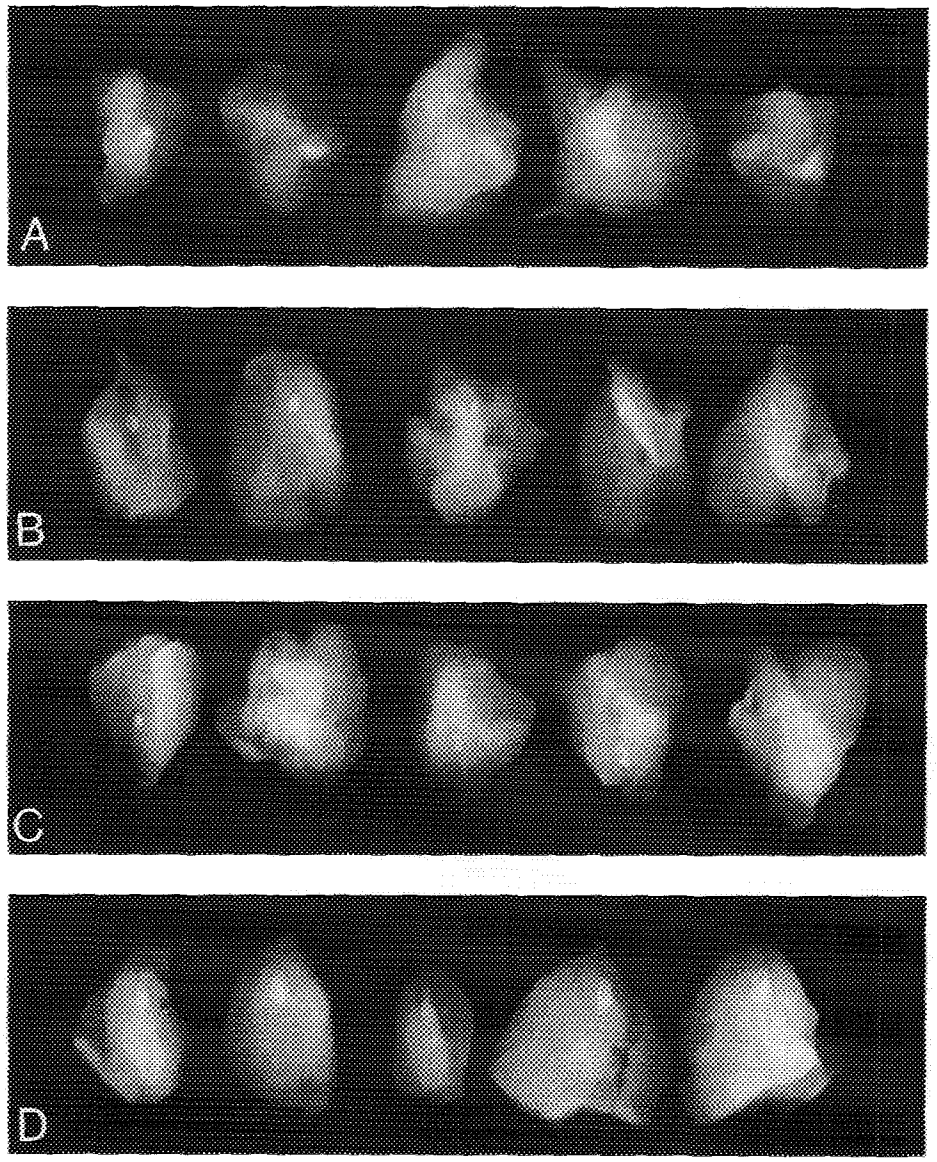

Figure 1. Soft X-ray photographs were taken four weeks after implantation. Radiopaque shadows were observed in each material which contained 2, 10, 50μg of rhBMP-2. (A) Control group, (B) 2μg, (C) 10μg, (D) 50μg.

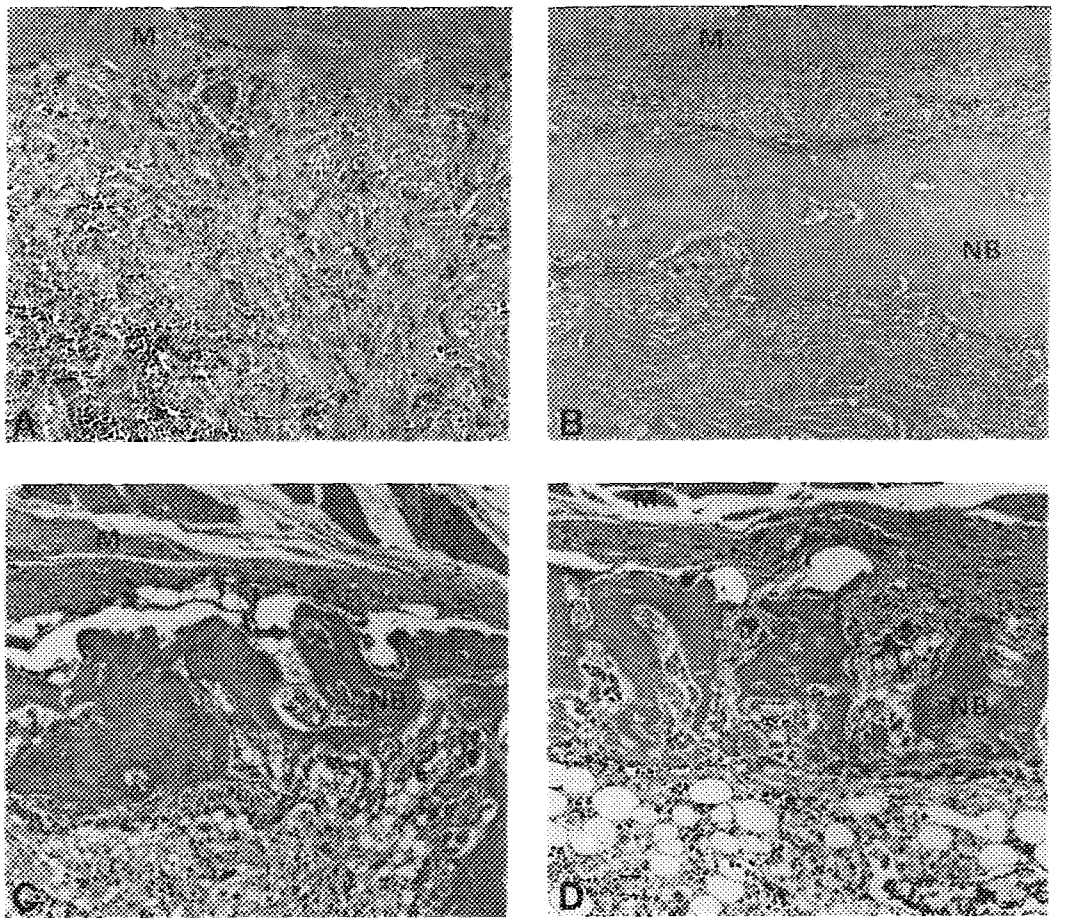

Figure 2. Photomicrographs of bone induced in calf muscle pouch of rats in groups Ⅱ, Ⅲ, Ⅳ. (A) Control group; There was no evidence of bone formation. (B) GroupⅡ; The trabecula bone tissue was found at the outermost edge of the implanted material. (C) GroupⅢ; The trabecula bone tissue was found on the outermost edge of the implanted material around almost the full circumference. (D) GroupⅣ; Trabecula bone tissue was found also deep within the implant area, and the resembling bone marrow and the angioid tissue were found in places (M: calf muscle of host; NB: newly formed bone; hematoxylin and eosin stain, X 84).

Histologic analysis: In each of the group Ⅱ, Ⅲ, Ⅳ, lightmicroscopic examination disclosed new bone formation in the calf muscles (Fig2), however, cartilage formation was never disclosed. Control group; there was no evidence of osteoinduction anywhere, and there was a part of the collagen pellet which remained in the nonabsobable form that was in part infiltrated by inflammatory cells. Group Ⅱ; there was trabecular bone in an area on the outermost edge of the implanted material adjacent to the host tissue. Group Ⅲ; there was

trabecular bone on the outermost edge of the implant material around almost the entire circumference, and evidence of osteoinduction by immature mesenchymal type cells surrounded the new bone. Group IV ; trabecular bone was observed not only on the outermost edge of the implant but also deep within the implant area. Osteogenesis was actively accomplished everywhere. The tissue of resembling bone marrow was indicated between trabecula bone, and angioid tissue which filled hemocytes was formed in places.

Quantitative analysis: The Ca content and ALP activity of each bioassay are shown (Fig3). The volume of bone formation tended to depend on the amount of rhBMP-2 implanted. Namely, 0.4 μg/mg (control group), 212.5 μg/mg (group II), 261.0 μg/mg (group III), and 368.0 μg/mg (group IV) were indicated on average of the Ca content. Under 5.0 IU/mg (control group and group II), 490.0 IU/mg (group III), and 1656.3 IU/mg (group IV) were indicated as the average of dose of the ALP activity.

DISCUSSION

In previous experiments about ectopic bone induction by BMP with carrier, demineralized and lyophilized animal bone(7-10), and sintered bone (true bone ceramic; TBC)(13), were reported. However, it is impossible to use large quantities of human or animal bone as a carrier clinically because of its production limitation. In addition, since the components of demineralized bone (with 4M guanidine-hydrochloride extracted) remain unknown, Bessho (14) first

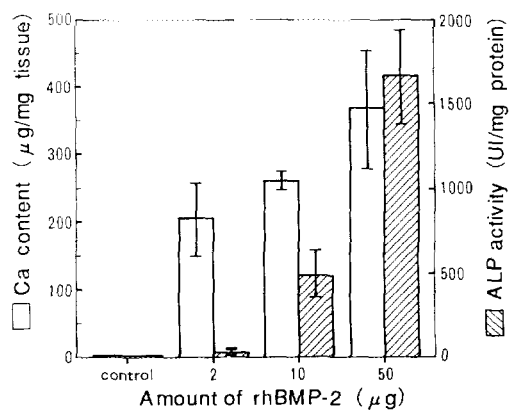

Figure 3. The results of determination of Ca content and ALP activity in each bioassay. The volume of bone formation tended to depend on the amount of rhBMP-2 implanted .

used the purier collagen which was used in our present study as a carrier for his highly purified BMP. He has used this collagen in his study since then (15). And it is possible to produce a large amount of this collagen in industry, however, it is derived from animals. Also, this collagen has a low immune response(16) and does not inhibit osteoinduction with BMP. Takaoka et al (17) also reported osteoinduction of composite material containing partially purified BMP from murine osteosarcoma and the collagen derived from bovine skin. On the other hand, because of suspecting its antigenicity or uncontrollable interstitial absorptive speed in vivo, they used polylactic acid-polyethlene glycol block copolymer as a carrier (18).

Recently the synthetic technology of rhBMP was established, and rhBMP will have been high frequency of use in clinically. So it would be necessary to use a mixture of rhBMP and some mass productive pure or artificial material as a carrier in future. In the many fields of medicine, recombinant bone morphogenetic factor such as rhBMP-2 will clinically has a great number of practical use. And in these clinical uses, the composited materials of osteoinductive factor and biodegradable carrier must be in a viscosity or in a plastic form using hydroxyapatite blocks. In orthodontics, however, it would be appropriate to use only a biodegradable carrier for reconstruction of the jaw, if the teeth near the implanted material are corrected in position, or if the patient is at the period of his/her jaw growth. The purpose of this study was to examine the ectopic osteoinductive activity of the mixture of rhBMP-2 and collagen derived from fresh porcine skin. Furthermore, this implanted materials was histologically studied to determine whether or not it is suitable for clinical use.

The results of this study indicate that atelopeptide type I collagen is effective and practicable as a carrier for rhBMP-2 in vivo sufficient to osteoinduct with even a small amount of rhBMP-2. And in further studies, because the volume of bone formation was differential in the quantity of implanted rhBMP-2 in histological findings, it is necessary to determine the appropriate implanting quantity of rhBMP-2 in the each region used, and to determine the proper blend ratio of collagen and rhBMP-2.

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