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Osteoinduction by Bone Morphogenetic Protein-2 via Adenoviral Vector under Transient Immunosuppression

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To examine the effectiveness of gene transfer of bone morphogenetic protein (BMP)-2 *in vivo*, we evaluated osteoinduction by an adenoviral vector, AxCAOBMP-2, under transient immunosuppression with an immunosuppression drug (cyclophosphamide), which was given at a dose of 125 mg/kg intraperitoneally the day before vector injection. Twenty-five microliters of AxCAOBMP-2 (8.75×10^8 pfu, Group I) and AxCALacZ (1.75×10^8 pfu, control group) and 5 μl of AxCAOBMP-2 (1.75×10^8 pfu, Group II) were injected into a right calf muscle. On day 21, induced bone in each group was investigated radiologically, histologically, and biochemically. The finding of osteoinduction was only seen in the AxCAOBMP-2-treated groups with immunosuppression. The activity of osteoinduction in Group I was higher than that in Group II. These results suggest that gene therapy with AxCAOBMP-2 under transient immunosuppression may be useful for bone reconstruction. © 2000 Academic Press

Bone morphogenetic proteins (BMPs) act on mesenchymal stem cells causing their differentiation into osteoblastic and chondroblastic pathway. These factors affect osteoblast and osteoclast metabolism in the bone tissue repair response. Thus, BMPs may have wide-ranging potential for clinical application. Since the synthesis of recombinant human (rh) BMP-2 (1), *in vivo* studies on the osteoinducing activity of rhBMP-2 have been performed (2–4).

Abbreviations used: BMP, bone morphogenetic protein; ALP, alkaline phosphatase; MOI, multiplicity of infection; AxCAOBMP-2, a replication-deficient adenoviral vector-carrying BMP-2 gene; AxCALacZ, a replication-deficient adenoviral vector-carrying the *Escherichia coli; E. coli, Escherichia coli;* X-gal, 5-bromo-4chloro-3-indolyl-β-D-(−)-galactopyranoside; pfu, plaque-forming units.

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Recently, gene therapy has been studied extensively, including clinical trials on cystic fibrosis were performed with viral vectors (5–9). Adenoviral vectors have highly efficient expression systems both *in vivo* and *in vitro* and can transduce the gene of interest even into nonreplicating cells (9, 10). There are some reports on the delivery of BMP genes (11–17), the application of osteoinduction using adenoviral vectors coding BMP-2 (10, 14–17). We have previously reported that gene transfer of BMP-2 into C2C12 myoblasts by an adenoviral vector *in vitro* converted these cells from myoblasts to osteoblast lineage (18). However, adenoviruses may induce an immune response that limits the duration of gene expression *in vivo* (17). Therefore, it was not able to control bone formation over time. The main reason for this is anti-adenoviral host immunity, including T-cell responses. Several methods have been used to overcome the T-cell responses, such as immunosuppression. Cyclophosphamide has been used primarily as an anticancer drug. This agent was found to be effective in blocking not only the T-cell mediated immunity but also the humoral response, i.e., anti-adenovirus neutralizing antibody (19, 20). In the present study, we evaluated osteoinduction by an adenoviral vector, carrying bone morphogenetic protein-2 gene under transient immunosuppression with cyclophosphamide.

MATERIALS AND METHODS

Construction of BMP-2 Expressing Recombinant Adenovirus

The details of construction were described previously (18). A replication-deficient type 5 adenoviral vector-carrying BMP-2 gene, AxCAOBMP-2, was constructed according to the cosmid cassette and adenoviral DNA terminal protein complex method (COS/TPC; Adenovirus Expression Vector Kit, Takara Shuzo Co., Ltd., Shiga, Japan), as previously described (21). This vector system with deletion of the E1 and E3 regions (22) contained the human BMP-2 gene under the control of a CAG promoter (chicken β-actin promoter and cytomegalovirus enhancer). The control vector was AxCALacZ (22) (a

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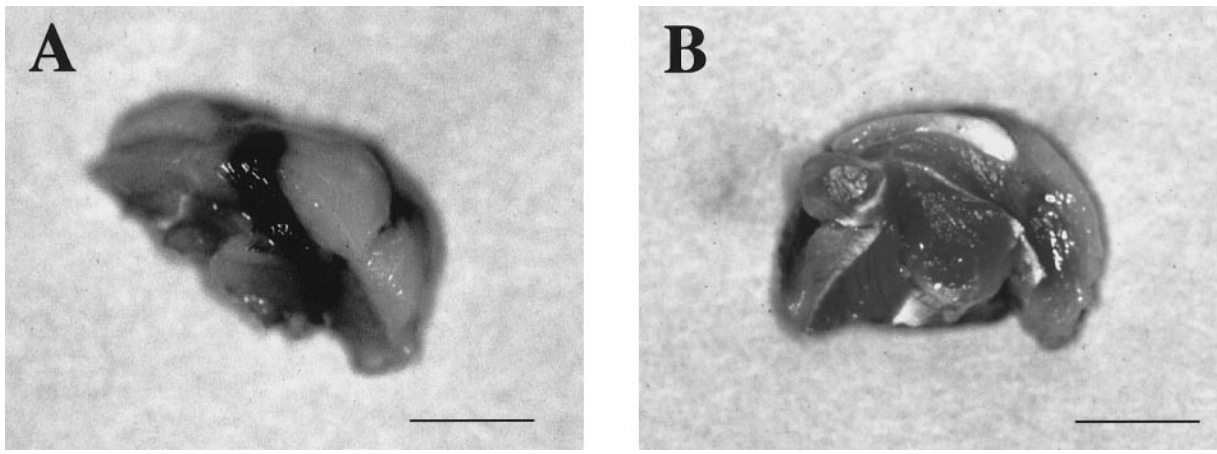

FIG. 1. X-gal staining of right calf muscles 3 days after treatment with AxCALacZ (A) and uninfected (B) (bar = 1 cm).

gift from I. Saito and Y. Kanegae), which carries the *Escherichia coli* (*E. coli*) lacZ cDNA in the same expression unit as AxCAOBMP-2. The viruses were titrated and stored in phosphate-buffered saline (PBS) containing 10% glycerol at −80°C (24).

In Vivo Study

Animals. Seventeen Wistar rats (male; 9 weeks old; weight, 230–250 g) were randomly assigned to 25 μl of AxCAOBMP-2 (8.75×10^8 pfu, Group I), 5 μl of AxCAOBMP-2 (1.75×10^8 pfu, Group II) or 25 μl of AxCALacZ (1.75×10^8 pfu, control group) of 5 rats each. The 2 remaining rats were used for X-gal staining. They were fed rodent chow (Certified diet MF; Oriental Koubo Inc., Tokyo, Japan) pre- and postoperatively. All procedures and virus inoculum were approved by the Institute of Laboratory Animals, Faculty of Medicine, Kyoto University.

Surgical procedure. Cyclophosphamide, provided by Shionogi & Co., Ltd. (Osaka, Japan), was given at a dose of 125 mg/kg injected intraperitoneally the day before vector injection. On the day of vector injection, all rats were anaesthetized by intraperitoneal administration of sodium pentobarbital (5.0 mg per 100 g of body weight). Following disinfection of the operative region, 25 μl of AxCAOBMP-2 and AxCALacZ, and 5 μl of AxCAOBMP-2 suspended with 20 μl of Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY) were injected into a right calf muscle with a microsyringe.

5-Bromo-4chloro-3-indolyl-β-D-(−)-galactopyranoside (X-Gal) histochemistry. To determine which cells were being transduced *in vivo,* AxCALacZ was injected into the calf muscle of one rat. In another rat, HBSS alone was injected into the calf muscle as a control. On day 3 postinjection, the 2 rats were anesthetized and underwent intracardial perfusion with fixative (2% paraformaldehyde, 5 mM ethylene glycol bis-*N*,*N*,*N*,*N*-tetraacetic acid, 2 mM $MgCl_2$ in 0.1 M pipes buffer). The right calf muscle was harvested and placed in fixative for 2 h and submerged in cold PBS. Samples were then placed in a substrate solution (containing 1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40) for 3 h. Cross-section samples of the calf muscles were taken from the center of the treated area. Then they were placed again in the substrate solution for 3 h and photographed.

Radiographic evaluation. Twenty-one days after the vector-injection, the rats were sacrificed with an overdose of sodium pentobarbital. Then the injection region was excised together with the surrounding tissue and soft X-rayed (SOFRON; SRO-M50, Sofron Inc., Tokyo, Japan). Each excised specimen was removed and cut into halves, one for histological analysis and the other for biochemical analysis.

Histological analysis. The specimens with peripheral tissue were fixed in 10% formalin neutral buffer solution (pH 7.4), demineralized in ethylenediamine tetraacetic acid, and embedded in paraffin. They were cut into 4-μm-thick sections and stained with hematoxylin and eosin.

For histomorphometric analysis, the trabecular area on histological micrographs on day 21 was measured on films using a computer system with Photoshop (Version 5.0J; Adobe, Mountain View, CA) and NIH image software (Version 1.58).

Biochemical examination. The samples were weighed and then homogenized in 0.25 M sucrose in a Polytron homogenizer (Bio-Mixer; type ABM, Nissei Inc., Osaka, Japan). The sediment was demineralized in 0.5 N hydrochloric acid, and the calcium (Ca) content of the soluble fraction was determined by the orthocresolphthalein complexone method (25). The alkaline phosphatase (ALP) activity and total protein in the resultant supernatant were determined by the 4-nitrophenylphosphate method (26). The Ca content (μg/mg of tissue) and the ALP activity (IU/mg of protein) were used as indices of bone formation.

Statistical analysis. The results are presented as mean ± standard errors of means (SEM). Statistical analysis of differences in the value of ALP activity, Ca content and histometry of microscopic views was performed by analysis of variance (ANOVA), followed by Fisher's comparison test.

RESULTS

All rats survived until the scheduled date of killing with no apparent complications.

X-gal Staining

AxCALacZ-infected calf muscle showed positive staining by X-gal reaction (Fig. 1A). However, the calf muscle without infection showed no staining (Fig. 1B).

Radiographic Findings

On day 21, soft X-ray revealed opaque shadows in Groups I and II (Figs. 2A and 2B). The oval shadows in Group I were larger with higher radio-opacity than

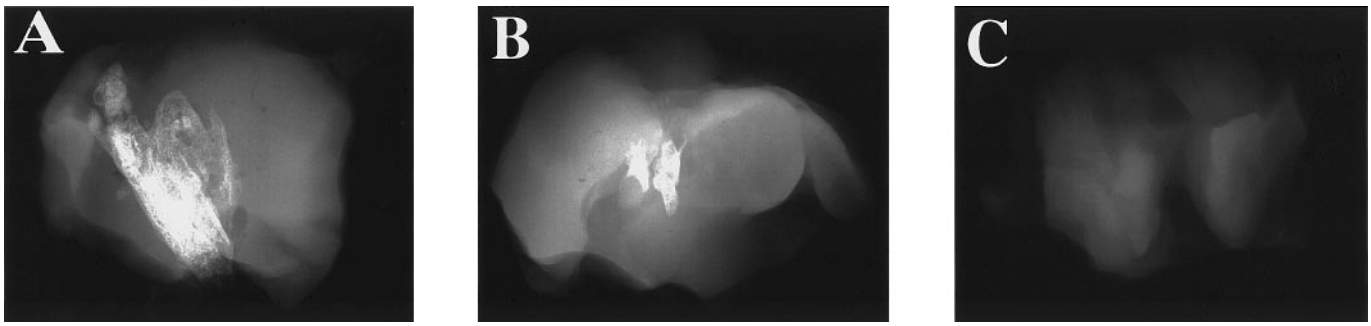

FIG. 2. Soft X-rayed film of calf muscles 21 days after treatment. (A) Group I, (B) Group II, (C) control group.

those in Group II. However, these radio-opaque images were not observed in the control virus-treated group (Fig. 2C) and also AxCAOBMP-2-treated without immunosuppression group (data not shown).

Histological Findings

No inflammatory responses were seen in any group. Light-microscopic examination disclosed new bone formation in Groups I and II (Figs. 3A and 3B). However, there was no evidence of osteoinduction in the control-virus-treated group (Fig. 3C). Around the trabecular bone, osteoclasts and lining osteoblasts were observed in both AxCAOBMP-2-treated groups. The number of osteoblasts and osteoclasts in Group I was greater than that in Group II. In Group I, the trabecular area was greater than that in Group II. The bone marrow area in Group I, including part of the fatty marrow, was wider than that in Group II. In both AxCAOBMP-2-treated groups, cartilage tissue was observed in the deep portion. The results of micrograph analysis of the trabecular area are summarized in Fig. 4A.

Biochemical Indices

ALP activity and Ca content on day 21 after the infection are shown in Figs. 4B and 4C, respectively. In the control-virus treated group or AxCAOBMP-2-treated without immunosuppression, ALP activity and Ca content were hardly elevated (data not shown). In both AxCAOBMP-2-treated groups, ALP activity and Ca content varied in a dose-dependent fashion. The values of ALP activity and Ca content in Group I were significantly higher than those in Group II.

DISCUSSION

In the present study, we constructed a BMP-2-expressing replication-deficient adenoviral vector, AxCAOBMP-2, and demonstrated that gene therapy can be used to lead to osteoinduction at the intramuscular site under transient immunosuppression with cyclophosphamide. In both AxCAOBMP-2-treated groups, new bone formation, including a considerable amount of trabecular bone, was detected on day 21 after infection. Prior to the gene transfer experiment with this AxCAOBMP-2 vector to calf muscle, we analyzed whether the adenoviral vector could transfer the gene of interest efficiently into calf muscle using AxCALacZ, which expresses the reporter lacZ gene. X-gal staining demonstrated that transgene expression occurs in calf muscle. In radiological, histological, and biochemical examinations, osteoinduction was confirmed in both AxCAOBMP-2-treated groups with immunosuppression. In the histological examination, the number of osteoblasts and osteoclasts in Group I was greater than that in Group II. In Group I, the trabecular area was significantly greater than that in Group

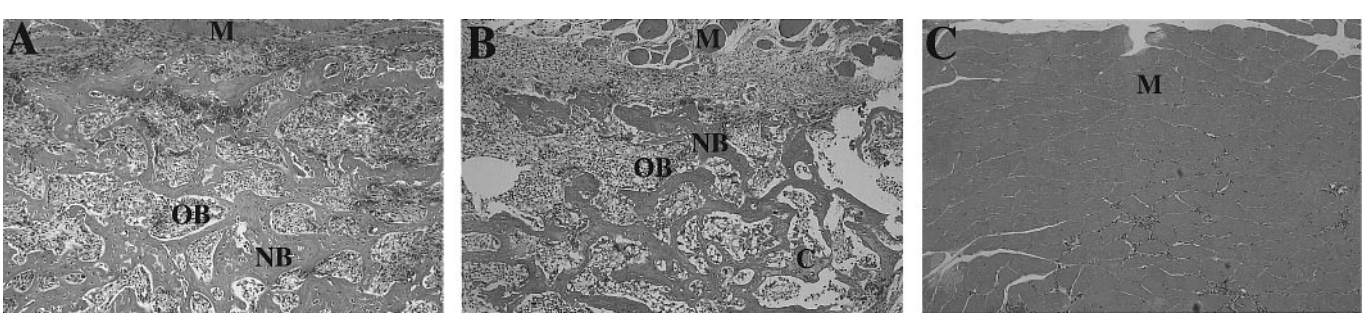

FIG. 3. Histological view of new bone formation 21 days after treatment (M, calf muscle of the host; C, cartilage tissue; NB, newly induced bone; OB, osteoblast; hematoxylin–eosin. Original magnification, × 25). (A) Group I, (B) Group II, (C) control group.

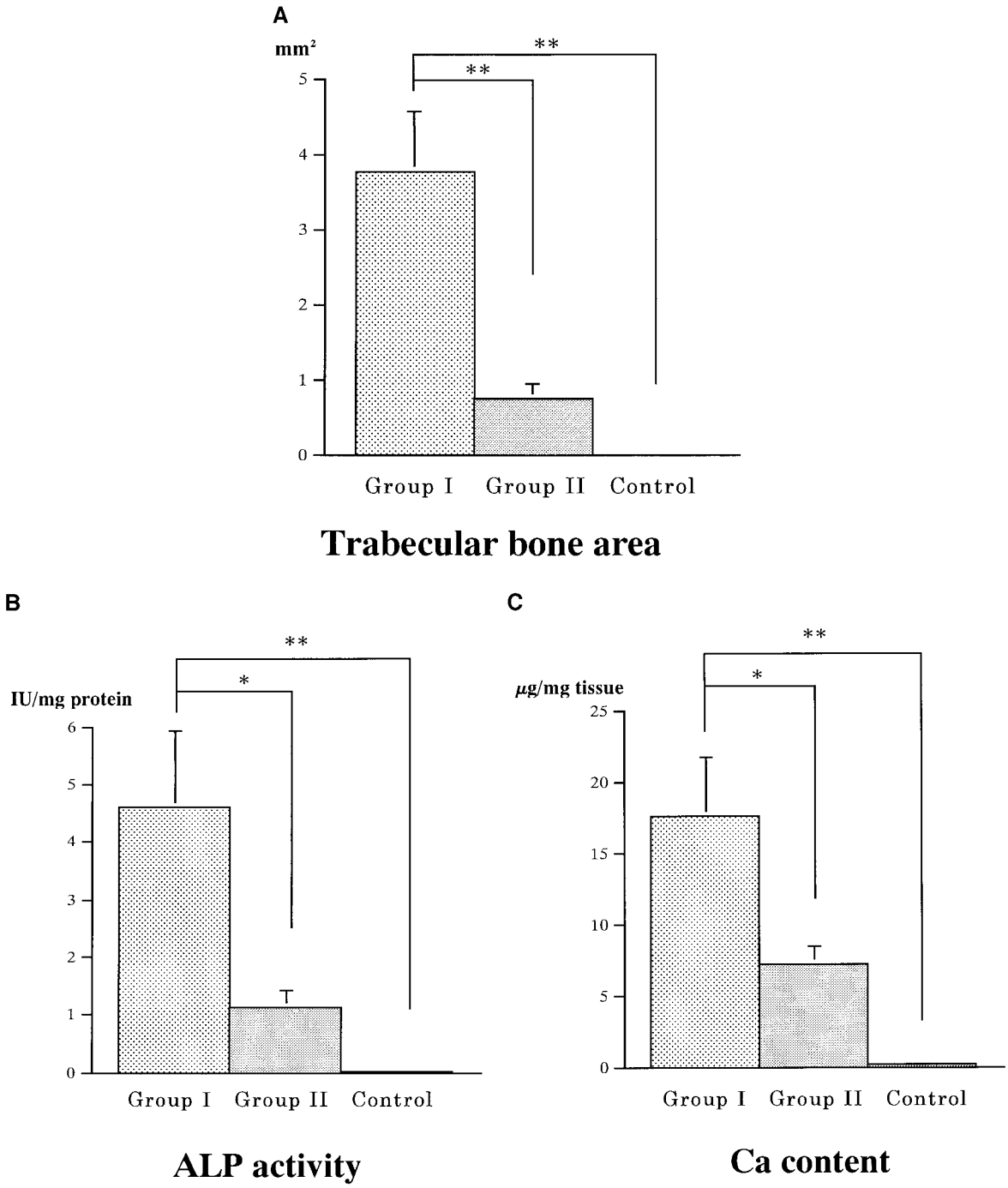

FIG. 4. The area of newly induced trabecular bone (A), values of ALP activity (B) and Ca content (C) 21 days after treatment. Data are means ± SEM. *$P < 0.05$, **$P < 0.005$.

II. These results suggested that the bone remodeling activity in Group I was higher than that in Group II. The values of ALP activity and Ca content in Group I were significantly higher than those in Group II.

In a previous study, ectopic bone formation *in vivo* is performed by the implantation of rhBMP-2 protein with a carrier that is derived from some living body because it is the most suitable carrier (2–4). Various types of carriers, such as collagen, have been evaluated *in vivo* studies. However, the disadvantage of this procedure is that surgical invasion is necessary. In clinical application, sufficient bone formation induced by rhBMP-2 protein can be difficult to obtain. Furthermore, these carriers are associated with problems,

such as antigenicity. Before gene therapy with AxCAOBMP-2, we confirmed that C2C12 myoblasts were converted into osteoblast lineage cells by gene transfer with AxCAOBMP-2. This method may be a more efficient way to deliver BMP-2 than protein delivery (18).

Adenovirus has advantages as a vector system in gene therapy in its ease of producing a high titer recombinant virus and high transduction efficiency, as well as its ability to transfer the gene of interest, even into non-dividing cells. Synthesis of adenoviral gene products which is leaked even from the E1-deleted adenovirus vector often stimulates an immune response to the infected cells and result in a loss of therapeutic gene expression 1–2 weeks after infection (27, 28). In addition, transferred genes are not integrated in the host chromosomes. Therefore, the adenoviral vector may be inappropriate for treating genetic disorders for which long-term gene expression is required, but instead, may be better suited for use in cardiovascular diseases and cancer. Osteoinduction by BMP-2 suits the latter case.

While, it was reported that bone formation by an adenoviral vector expressing BMP-2 was detected in nude rats but not in immunocompetent rats (17). In the X-gal staining in this study, there was very little difference in the expression of LacZ between immunosuppressed rats and normal rats on day 3 postinjection (data not shown). However, osteoinduction was not seen in normal rats (data not shown). We suspected that the reason for this was that the infected cells were eliminated by the immune system of the host after day 3 postinjection. $CD8^+$ and $CD4^+$ T-cells have been shown to play a primary role in this decreased gene expression (28, 29). To overcome T-cell responses, several methods have been used, such as inhibiting the CD28/B7 pathway (30), blocking NF-κB activation (31), inhibiting tumor necrosis factor α activity (32), administering an anti-CD4 antibody (33), and immunosuppression (34, 35). Cyclophosphamide is used clinically as an anti-cancer agent in the treatment of Hodgkin's disease and other leukemias. This agent is known to effectively block humoral immunity and inhibit the cellular immune system. Cyclophosphamide, administered at a dose of 300 mg/kg the day before vector injection in mice, was effective in blocking the humoral response and enhanced the effectiveness of the second injection with the adenoviral vector (20). In this study model, readministration of AxCAOBMP-2 may be also possible. Therefore, gene therapy with AxCAOBMP-2 may also control bone formation over time by readministration of AxCAOBMP-2.

The current results suggest that gene therapy with AxCAOBMP-2 under transient immunosuppression may have potential applications for the treatment of several genetic disease and bone reconstruction.

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