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Heterotopic Ossification of Degenerating Rat Skeletal Muscle Induced by Adenovirus-Mediated Transfer of Bone Morphogenetic Protein-2 Gene*

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ABSTRACT

In vivo gene transfer is a recently developed device for efficient delivery of a therapeutic recombinant protein. We formulated the hypothesis that a high level of expression of bone morphogenetic protein 2 (BMP-2) could be a future therapeutic modality in terms of inducing substantial bone formation in vivo. First, to test this hypothesis, adenoviruses carrying *BMP-2* gene were directly injected into the soleus muscle of adult rat. The *BMP-2* gene was successfully overexpressed in the target muscle by adenovirus-mediated transfer, whereas bone formation in and around the muscle failed to occur in this case. Second, to recruit putative osteoprogenitor cells, we then induced ischemic degeneration of the target muscle by orthotopically grafting it simultaneously with the gene transfer. The combination of *BMP-2* gene transfer and orthotopic muscle grafting resulted in successful ossification of almost the whole grafted muscle, whereas neither muscle grafting alone nor the combination of muscle grafting and adenovirus-mediated transfer of reporter gene *LacZ* induced any bone formation in the muscle. The ossification process was evident by positive von Kossa staining of the histological sections and roentgenographical radio-opacity of the region. It was also found that the *BMP-2* transgene overexpressed in grafted muscles inhibited muscle regeneration, which should otherwise follow the muscle degeneration. We further demonstrated an up-regulation of BMP receptor type IA in grafted muscles, suggesting its involvement in the bone-formation process. In conclusion, overexpression of *BMP-2* gene induced massive heterotopic ossification in skeletal muscles under graft-induced ischemic degeneration, which possibly up-regulates osteoprogenitor cells in situ. (J Bone Miner Res 2000;15:1056–1065)

Key words: bone morphogenetic protein 2 (BMP-2), adenovirus, gene therapy, skeletal muscle, ossification

INTRODUCTION

REPAIR OF SKELETAL bone defects remains a challenging problem for orthopedic, plastic, and reconstructive surgeons. Although a variety of surgical modalities including autogenous[1,2] or allogenic[2] bone grafting, biocompatible synthetic material grafting,[3] and distraction callostasis of Ilizarov[4,5] are presently available, there are still problems and limitations associated with each method.[6] A multidisciplinary approach is therefore needed to develop a more successful modality for bony reconstruction.

An approach for inducing autogenous osteogenesis in situ, which has been utilized since reported by Urist,[7] continues to show promise. Such an approach includes

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autogenous osteoblast transplantation and the implantation of synthetic materials combined with bioactive molecules such as bone morphogenetic protein (BMP).[6] Some members of the BMP family are considered likely to induce new bone formation at the sites of bone nonunion and extensive bone defects in humans.[8,9] It is known that a simple implantation of purified, water-soluble BMP alone induces no local bone formation in vivo,[10] probably because the protein is promptly diffused, degraded, or dispersed from the implantation site. In most of the successful experiments,[11–14] BMP protein was implanted together with an adequate carrier matrix to maintain a certain local concentration. However, bone formations evoked by BMP protein were limited only within the area that the carrier matrix occupied, or a little beyond it at most, and appeared to be insufficient for therapeutic purposes.

Gene therapy is an efficient delivery system for diverse recombinant proteins in vivo.[15] We formulated the hypothesis that a high level of expression of BMP-2, a strong osteoinductive molecule, induces substantial bone formation in vivo. Upon this hypothesis, we developed an adenovirus-mediated transfer system of the human *BMP-2* gene, aiming at the high BMP-2 expression in vivo. We adopted skeletal muscles as the target of gene transfer, because they have been reported to be amenable to adenovirus-mediated gene transfer.[16] After multiple trials seeking for the optimal condition of the BMP-2-induced bone formation in vivo, we finally found that *BMP-2* gene transfer into grafted muscle resulted in substantial ossification in the target region. This ossification far exceeds that reported in past studies, which also utilized BMP-2 in terms of its magnitude and its extent. Furthermore, we examined the temporal and spatial pattern of expression of BMP-2 receptors in surgically manipulated muscles. As a result, we found that the BMP receptor was up-regulated by a muscle-grafting procedure. Since skeletal muscles could easily be transplanted to a diseased portion surgically, the study presented here also suggests the possible clinical implications of ossification introduced into the surgically grafted skeletal muscle by *BMP-2* transgene overexpression.

MATERIALS AND METHODS

Gene construction of an adenoviral vector

A replication-deficient adenovirus vector carrying the CMV-IE enhancer, chicken β-actin promoter, and the coding region of human *BMP-2* (AxCABMP2) was constructed as described before[17] (Fig. 1). Briefly, an expression unit containing the coding region of *BMP-2* was cloned into a cosmid, pAxcw. The resulting cosmid, pAxCABMP2, was cotransfected into the 293 embryonic cell line with an *Eco*T221-digested DNA-TPC (from Ad5dlx) to generate AxCABMP2. The obtained viruses were isolated, screened for the BMP-2 insert, and then propagated. A replication-deficient recombinant adenovirus carrying the *Escherichia coli β-galactosidase* gene AxCALacZ was kindly provided by Dr. I. Saito.[18] For in vivo application, the viruses were propagated, purified, and titrated as described.[19]

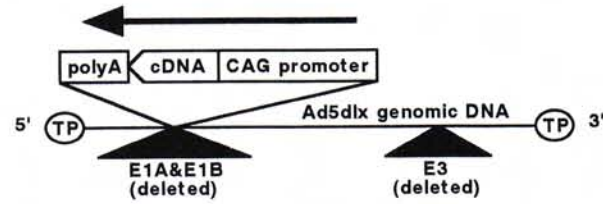

FIG. 1. Genomic DNA construction of adenoviral vector encoding human *BMP-2* and *LacZ*. CAG promotor, CMV-IE enhancer and chicken β-actin promoter; TP, Ad5 virus terminal protein. Human *BMP-2* or *LacZ* cDNA with CAG promoter and polyA were inserted into replication-deficient adenoviral vector Ad5dlx. The direction of transcription is shown by an arrow.

Animals

Eight-week-old specific pathogen-free male Wistar rats weighing 250–300 g were used as in vivo experimental models. All procedures were performed according to the Guide for Animal Experimentation of the University of Tokyo. Operations were performed under sterile conditions and general anesthesia was induced by the intraperitoneal and intramuscular administration of ketamine hydrochloride (90 mg/kg body weight) and xylazine (15 mg/kg body weight), respectively. Ketamine hydrochloride was supplemented as necessary. Unrestricted and weight-bearing activity was allowed postoperatively.

In vivo gene transfer into skeletal muscle

We utilized the soleus muscle of the rat right hindlimb as a target for in vivo gene transfer. After exposing its surface through an open incision, a 50-μl aliquot of an adenoviral solution containing 5×10^8 plaque-forming units (pfu) of AxCABMP2, AxCALacZ, or Ad5dlx was injected into the muscle belly using a 27-gauge needle under the operative microscope, taking care not to allow it to leak out of the muscle (BMP-muscle group or LacZ-muscle group, respectively). When the leakage of injected adenoviral solution was found, the animal was excluded from the experimental group. In some animals, *BMP-2* and *LacZ* gene transfers into the soleus muscles were performed simultaneously with the orthotopic grafting (BMP-graft group and LacZ-graft group, respectively). At least four animals at each time point for each group were operated on and used for histological examinations.

Orthotopic muscle grafting

The soleus muscle of rat right hindlimb was grafted orthotopically to elicit ischemic degeneration followed by regeneration as described elsewhere.[20] Briefly, under an operative microscope (Carl Zeiss, Germany), the soleus muscle was isolated, followed by transection of both tendons. Its nutrient vessels were transected with the neural pedicle preserved intact. Consequently, vascular supply to the soleus muscle was blocked without denervation and the

TABLE 1. OLIGONUCLEOTIDE PRIMERS USED IN THE PCR AMPLIFICATION OF BMP-2

Gene	*Primer sequence*[a]	*GenBank accession no.*	*Nucleotide coordinates*
human *BMP-2*	CCACGGAGGAGTTTATCACC	M22489	742–761
	CAAAAGTTACTAGCAATGGC		1125–1106
GAPDH[b]	CTGCACCACCAACTGCTTAGC	M17701	477–497
	CTCAGTGTAGCCCAGGATGCC		858–838

[a] All primer sequences are written from 5′ to 3′. For each primer pair the top sequence is sense and the bottom sequence is antisense.
[b] GAPDH is rat glyceraldehyde-3-phosphate dehydrogense and was used as a positive control.

muscle underwent ischemic degeneration. Both tendons were subsequently sutured to the same place with 6-0 Vicryl (Ethicon, Inc., Somerville, NJ, U.S.A.) without any microvascular anastomosis of the transected nutrient vessels. Some rats underwent orthotopic grafting of their right soleus muscles alone (Muscle-graft group, $n = 3$ at each time point), and other rats underwent the grafting in conjunction with the *BMP-2* or *LacZ* gene transfer (BMP-graft group or LacZ-graft group, respectively, $n \geqq 4$ at each time point).

β-Galactosidase activity assay

Transfected muscles were harvested 5 days after the gene transfer, when the expression of β-galactosidase reached a maximum. They were homogenized in a lysis buffer and further assayed using a β-Galactosidase Enzyme Assay System (Promega, WI, U.S.A.) according to the manufacturer's instructions. The protein concentration of each sample was also determined using a Micro BCA Protein Assay Reagent Kit (Pierce, IL, U.S.A.).

RT-PCR analysis

Total RNA was obtained from a whole soleus muscle homogenate with the acid guanidium thiocyanate/phenol/chloroform extraction technique.[21] To identify the expression of human *BMP-2* gene transferred into the soleus muscles, we performed RT-PCR with *BMP-2*-specific primers. A reverse-transcriptase reaction was performed using 10 μg of total RNA in a 40-μl reaction mixture (final concentrations: 50 mM Tris hydrochloride, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM dNTP, pH 8.3) containing 200 U of Superscript II (Life Technologies, Inc., Rockville, MD, U.S.A.) at 37°C for 60 minutes, followed by inactivation of the enzyme at 70°C for 10 minutes. Control reaction was performed in parallel with an otherwise identical reaction without the reverse transcriptase. Gene-specific oligonucleotide primers were synthesized on the basis of the published human *BMP-2*[22] and rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sequences[23] (Table 1). The primer sequences for *BMP-2* were selected from the regions with low sequence homology between the human and rat *BMP-2* cDNAs[24] to prevent excrescent PCR amplification of the latter. The PCR reaction was comprised of 40 cycles, consisting of denaturation at 94°C (30 s), annealing at 57°C (1 minute), and extension at 72°C (2 minutes), in a total volume of 50 μl in a standard condition. PCR products were separated on 1.2% agarose gels.

Histological examination and immunohistochemistry

The harvested muscle specimens were embedded in OCT-compound (Miles, Inc., IL, U.S.A.) and rapidly frozen for sectioning. The anti-BMP type I receptor A and B (BMPR-IA and -IB) polyclonal antibodies[25,26] were kind gifts from Dr. Kohei Miyazono. Monoclonal antibodies, F1.652, which reacts with embryonic and fetal myosin heavy chain,[27] anti-β-galactosidase clone BG-O2,[28] and anti-Dystrophin (carboxy terminus) clone DYS2 (Dy8/6C5)[29,30] were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, IA, U.S.A.), SAN-BIO b.v. (Amsterdam, The Netherlands), and Novocastra Laboratories Ltd. (Newcastle, U.K.), respectively. Immunostaining was performed using ABC *Elite* Kit (Vector Laboratories, Burlingame, CA, U.S.A.) according to the manufacturer's instructions. For reactions with primary antibodies, frozen cross sections, 5 to 8 μm thick, blocked with 4% skim milk were incubated with anti-β-galactosidase (1:50 dilution), anti-Dystrophin (1:4 dilution), anti-BMPR-IA (1.1 μg/ml), anti-BMPR-IB (1.0 μg/ml), or F1.652 (16 μg/ml) overnight at 4°C. Some sections stained with anti-BMPR-IA were counterstained with methyl green to determine the total cell nucleus number. For double-staining of BMPR-IA and Dystrophin, BMPR-IA was first labeled using VECTOR SG Substrate Kit for Peroxidase (Vector Laboratories) and then Dystrophin was labeled using VECTOR VIP Substrate Kit for Peroxidase (Vector Laboratories) according to the recommended protocol for double-label immunostaining.

To quantify the expression of embryonic/fetal myosin heavy chain, the cross-sectional areas of positive fibers were measured. The whole views of two F1.652-stained sections for each sample were photographed and processed using Adobe Photoshop 3.0, and then the cross-sectional areas were determined using image analyzing software, NIH Image (version 1.59).

To determine the population of BMPR-IA-positive cells, four or five fields were randomly selected for each cross section stained with anti-BMPR-IA and methyl green, and the numbers of positively stained cells were counted. As an internal control for BMPR-IA-positive cells, methyl green–stained nuclei were also counted. At least 1000 nuclei in all were counted for each specimen.

Statistical analysis

All data are presented as means ± SD. The data were statistically analyzed using Kruskal–Wallis nonparametric test followed by Scheffe's F multiple-comparison test. Differences in the percentages of cross-sectional areas of embryonic/fetal myosin heavy-chain-positive fibers between groups of the same time point were tested using Mann–Whitney's U test. A value of $p < 0.05$ was considered significant.

RESULTS

Induction of heterotopic ossification in grafted muscle by adenovirus-mediated transfer of BMP-2 gene

We first tried to induce bone formation in rat soleus muscle by intramuscular injection of AxCABMP2 adenoviral solution, which was reported to be an efficient administration route for adenovirus-mediated gene transfer into skeletal muscle.[16,31] However, bone formation never ensued in the injected muscles by this method (see below). Therefore, we examined the transgene overexpression by intramuscular injection of adenoviral solution, using AxCALacZ, the replication-deficient adenovirus encoding β-galactosidase. The β-galactosidase activities, measured 5 days after the injection of adenoviral solution, of *LacZ* gene-transferred soleus muscles (LacZ-muscle group) were 313 ± 106 mU/mg protein, whereas those of untransfected muscles and empty-vector Ad5dlx-transfected muscles were at background levels (0.321 ± 0.570 and 0.964 ± 1.12 mU/mg protein, respectively; Fig. 2A), indicating that our system of adenovirus-mediated gene transfer functioned efficiently. We also confirmed the overexpression of the *BMP-2* transgene in *BMP-2* gene-transferred muscles with RT-PCR analysis. Following RT-PCR using the human *BMP-2*-specific primer pair, the predicted 384-bp product was amplified from the muscles of the BMP-muscle group but not from untransfected muscles (Fig. 2B).

It has been reported that osteoinductive factor–induced heterotopic ossification requires the recruitment of osteoprogenitor cells.[32] Together with the failure of bone formation in AxCABMP2-infected muscles, we speculated that recruitment of osteoprogenitor cells, besides the expression of *BMP-2*, would be indispensable to induce the formation of new bone in skeletal muscle. However, injection of precursor cells was not thought to be practically feasible because the tissue-engineering technique including autogenous implantation of marrow stromal cells appears to be inefficient for mesenchymal cell recruitment. Therefore, we tried to recruit such precursor cells by either intramuscular injection of the local anesthetic bupivacaine,[33] direct contusion of the muscle,[34] or ischemic degeneration induced by orthotopic muscle grafting,[20] all of which are reported to induce mesenchymal cell proliferation.

We then examined the effect of transfection of AxCABMP2 in these surgically manipulated muscles and found that ossification of the muscle occurred only when AxCABMP2 was injected into the grafted muscles (BMP-graft group). In this case, the grafted muscles once underwent ischemic degeneration and then a large part of the soleus muscle had transformed into bone by day 28, whereas ossification was not detected in the neighboring skeletal muscles. The efficiency of gene expression through adenovirus-mediated gene transfer was assessed using AxCALacZ. The β-galactosidase activities in the LacZ-graft group (502 ± 17.8 mU/mg protein) were significantly higher than those in the LacZ-muscle group (Fig. 2A), indicating that the higher gene transfer efficiency in grafted muscles is one of the factors in successful bone formation. Also, RT-PCR analysis for the human *BMP-2* gene demonstrated the transgene expression in the muscles of the BMP-graft group (Fig. 2B).

We examined types of the transfected cells by immunolocalizing the recombinant β-galactosidase in AxCALacZ-transfected muscles. In the LacZ-muscle group, β-galactosidase was expressed in small cells that were localized along myofiber endomysia but not in mature myofibers. Most β-galactosidase-expressing cells expressed embry-

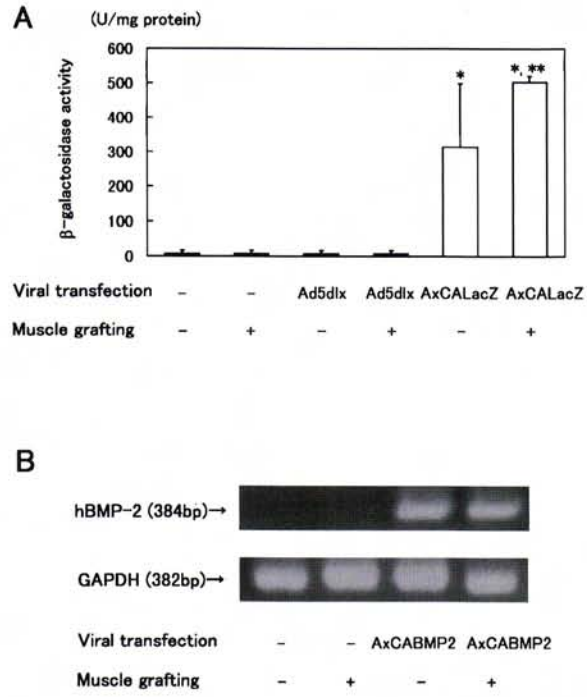

FIG. 2. Expression of transgenes in rat skeletal muscles by adenovirus-mediated gene transfer. (A) Activities of β-galactosidase, the reporter gene *LacZ* transcript, in untransfected, empty-vector Ad5dlx-transfected and AxCALacZ-transfected muscles. Values are means ± SD of four specimens per group of animals. * $p < 0.01$ versus no transfection and mock transfection; ** $p < 0.01$ versus AxCALacZ-transfected ungrafted muscle group. (B) Reverse transcription (RT)-PCR analysis of human *BMP-2* and rat *GAPDH* expression in untransfected and *BMP-2* gene-transferred muscles. Reverse-transcribed cDNA from the muscle sample of each group was subjected to PCR using *BMP-2* and *GAPDH*-specific primer pairs. These findings were confirmed in two separate experiments.

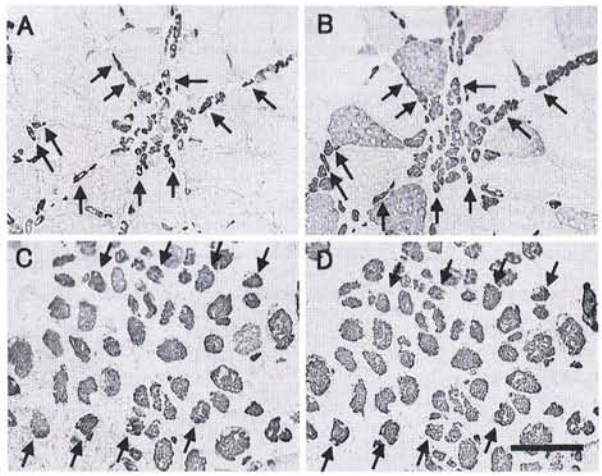

FIG. 3. Similar distribution of the expressed β-galactosidase and embryonic/fetal myosin heavy chain in *LacZ* gene-transferred rat skeletal muscles. Ungrafted (A, B) and grafted (C, D) soleus muscles were transfected with AxCA-LacZ and harvested 7 days after transfection. Serial sections were immunostained with anti-β-galactosidase (A, C) and antiembryonic/fetal myosin heavy chain (B, D). (A) Small cells along myofiber endomysiums (arrows), but not mature myofibers, expressed β-galactosidase. (B) Most of these small cells (arrows) also expressed embryonic/fetal myosin heavy chain, indicating that they were activated satellite cells. (C) Regenerating myofibers (arrows) expressed β-galactosidase. (D) Most of these fibers (arrows) also expressed embryonic/fetal myosin heavy chain, indicating that they were regenerating fibers. Bar, 100 μm.

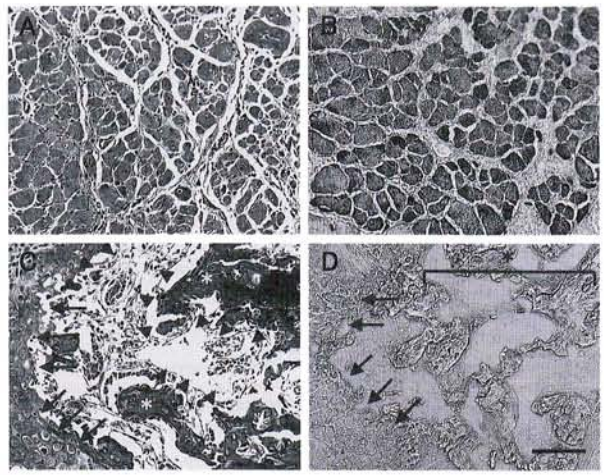

FIG. 4. Inhibition of graft-induced regeneration in *BMP-2* gene-transferred rat skeletal muscles. Muscles were orthotopically grafted and simultaneously transfected with Ax-CALacZ (A, B) or AxCABMP2 (C, D), and were harvested 14 days after grafting/gene transfer. Cross sections of soleus muscles were stained with hemotoxylin and eosin (H&E) (A, C) and immunostained with antiembryonic/fetal myosin heavy chain (B, D). (A) Most of the myofibers were in regenerating phase. (B) Embryonic/fetal myosin heavy chain was expressed in all regenerating fibers. (C) Degenerating myofibers have been replaced by cartilage (arrows) and trabeculae of mineralized bone (asterisks) with marrow sinuses. Note colonies of hematopoietic cells in the marrow sinuses (arrowheads). (D) Expression of embryonic/fetal myosin heavy chain, and thus myofiber regeneration, are suppressed both in the cartilage zone (arrows) and in the ossified zone (asterisk). Bar, 100 μm.

onic/fetal myosin heavy chain, strongly suggesting that they were activated satellite cells (Figs. 3A and 3B). β-Galactosidase was expressed in the cytoplasm of regenerating fibers, which characteristically expressed embryonic/fetal myosin heavy chain in the LacZ-graft group (Figs. 3C and 3D). Because satellite cells fuse with each other and form new regenerating myofibers,[35] it is suggested that β-galactosidases expressed in the cytoplasm of regenerating fibers are derived from transfected satellite cells involved in the muscle regeneration.

Suppression of muscle regeneration in BMP-graft group

Regenerative changes of rat soleus muscles caused by orthotopic grafting have been described elsewhere.[20] Briefly, almost all existing myofibers in the soleus muscle go into ischemic degeneration and the degenerated fibers are phagocytized by macrophages by postoperative day 3. Satellite cells, proliferation-potent myoblasts resident in the muscle, rapidly proliferate and differentiate into regenerating myofibers to reconstitute the degenerated muscle. In normal skeletal muscles of 8-week-old rats, adult isoforms are the predominant phenotypes of myosin heavy chain, and the embryonic/fetal isoform is usually not detected. Skeletal myofibers in the regenerating phase have been reported to undergo a change in the expression of myosin heavy-chain isoforms, that is, temporal expression of the embryonic/fetal isoform and progressive transition to the adult isoforms.[27] Embryonic/fetal myosin heavy chain was expressed in all regenerating fibers on day 7 through day 14 in the Muscle-graft group (data not shown).

The gross appearance and the patterns of embryonic/fetal myosin heavy-chain expression did not differ between the muscles in the Muscle-graft (data not shown) and LacZ-graft groups (Figs. 4A and 4B). In contrast, the muscle regeneration and the expression of embryonic/fetal myosin heavy chain of the grafted muscles were remarkably suppressed in the BMP-graft group throughout the experimental period (Figs. 4C and 4D). Quantification by image analysis revealed that embryonic/fetal myosin heavy-chain-positive fibers significantly decreased in the BMP-graft group compared with the LacZ-graft group on days 7 and 14 (Table 2). The difference between the *BMP-2* gene-transferred and *LacZ* gene-transferred muscles was less conspicuous on day 14 than on day 7. The reason for this is not clear; however, it was noted that some cells in the area surrounding the bone formation strongly expressed embryonic/fetal myosin heavy chain in the *BMP-2* gene-

TABLE 2. PERCENTAGE OF CROSS-SECTIONAL AREAS OF EMBRYONIC/FETAL MYOSIN HEAVY-CHAIN–POSITIVE REGENERATING FIBERS TO THOSE OF WHOLE MUSCLE IN ORTHOTOPICALLY GRAFTED RAT SOLEUS MUSCLES

Gene transfer	*Day 5*	*Day 7*	*Day 14*
LacZ	9.4 ± 8.6	35.1 ± 4.7	56.1 ± 8.4
BMP-2	8.8 ± 5.9	4.1 ± 2.0*	13.1 ± 20.0*

Rat soleus muscles were transfected with an adenoviral vector carrying LacZ or BMP-2 gene (each $n = 12$) and simultaneously grafted orthotopically. The animals were killed sequentially at days 5, 7, and 14 ($n = 4$ at each time point in each transfection group). Muscle specimens were cross-sectioned and two sections of each specimen were immunostained with F1.652, antiembryonic/fetal myosin heavy-chain monoclonal antibody. The cross-sectional areas of both the whole muscle and F1.652-positive regenerating fibers were measured using an image-analyzing software (NIH-Image). Cross-sectional areas of regenerating fibers are expressed as percentages to those of the whole muscle. Values are means ± SD of four specimens per time point and per group of animals.

* Significance of difference from the value for the LacZ-transfer group, $p < 0.05$.

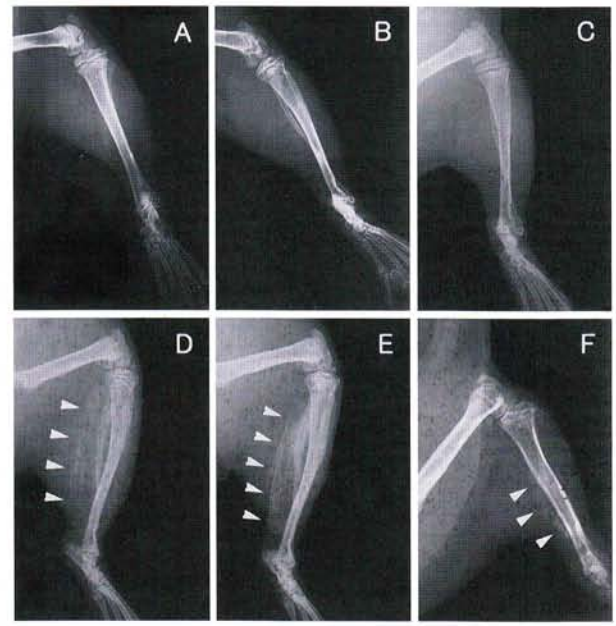

FIG. 5. Extraskeletal bone formation in rat hindlimb, where the soleus muscle was transferred with *BMP-2* gene, X-ray photographs. An aliquot of adenoviral solution of empty-vector Ad5dlx (A, B) and AxCABMP2 (C–F) was injected into the muscle, either alone (A, C) or simultaneously with orthotopic muscle grafting (B, D–F). (A–D) were taken 7 days after transfection; (E), 28 days after transfection; and (F), 1 year after transfection. (A–C) No extraskeletal radio-opaque regions can be seen. (D–F) Radio-opaque regions, suggesting bone formation, are clearly observed in the areas corresponding to grafted soleus muscles (arrowheads).

transferred muscle on day 14, when its expression reaches maximum after muscle grafting. These areas, abundant in embryonic/fetal myosin heavy chain in BMP-graft group muscles, were replaced by massive bone tissue finally by day 28 as were the early-ossified areas. These results suggest that the *BMP-2* transgene expression, but not the viral infection, is responsible for the inhibition of muscle regeneration caused by grafting.

Characterization of soleus muscle ossification

Heterotopic ossification occurred only when the *BMP-2* transgene was overexpressed in grafted muscle (BMP-graft group). Roentgenographical examination clearly showed radio-opaque regions, suggesting bone formation, in all the corresponding grafted muscles examined from day 7 through day 28 (Figs. 5D and 5E). In contrast, no bone formation was observed roentgenographically and histologically in either untransfected *grafted* muscle (Muscle-graft group, not shown), empty-vector-transfected *ungrafted* (Fig. 5A) and *grafted* (Fig. 5B) muscles, *LacZ* gene-transferred *ungrafted* (LacZ-muscle group, not shown), or *grafted* muscles (LacZ-graft group, Fig. 6A), or *BMP-2* gene-transferred *ungrafted* muscles (BMP-muscle group, Figs. 5C and 6B) up to day 28. Macroscopically, the soleus muscle, except for scattered regions of regenerating myofibers, had ossified and increased in volume by 2- to several-fold by day 7 in the BMP-graft group, whereas the neighboring skeletal muscles of the hindlimb were not affected, except for being stretched by the ossified soleus. The histological sections also showed the features of endochondral ossification at day 7, that is, a cartilage zone formation, its hypertrophy, vascular invasion, and the generation of trabeculae of mineralized bone (Fig. 6C). By day 28, a large part of the grafted muscle was completely transformed into bone. It is noteworthy that a thick cortex with vast marrow sinus was formed on day 28 (Fig. 6D). This ossified region was confirmed roentgenographically even 1 year after the gene transfer, although it gradually decreased in volume (Fig. 5F).

Expression of BMPR-IA in soleus muscle

In the sections of normal soleus muscle, BMPR-IA-positive cells were localized along myofiber endomysia (Fig. 7A), suggesting that they were predominantly satellite cells. Unfortunately, there is no specific biochemical marker for satellite cells in quiescent normal muscle, and therefore they can be identified only by anatomical localization, that is, localization between the plasma membrane and basement membrane. We then double-stained the normal soleus muscles with BMPR-IA and Dystrophin, a protein that is expressed on the plasma membrane of myofibers. BMPR-IA-positive cells were localized outside the plasma membranes that were stained by anti-Dystrophin, strongly suggesting that they were satellite cells (Fig. 7B). BMPR-IA-positive cells were also found in the connective tissue around the intramuscular small vessels (Fig. 7C), indicating that some of them were perivascular mesenchymal cells.[36]

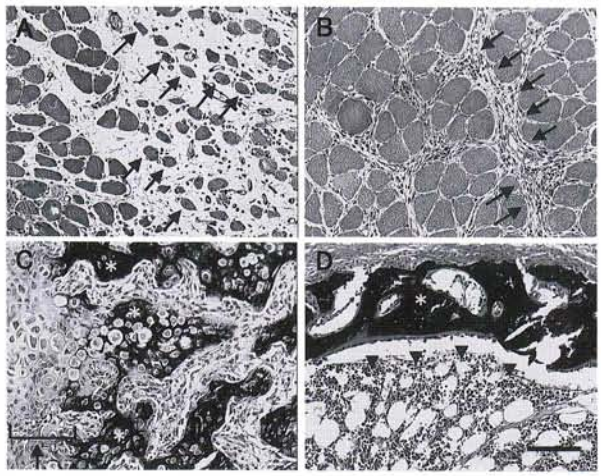

FIG. 6. Induced bone formation in *BMP-2* gene-transferred grafted rat soleus muscle. Muscles were transfected with AxCALacZ (A) and AxCABMP2 (B–D), either alone (B) or simultaneously with orthotopic grafting (A, C, D). They were harvested 7 (A–C) or 28 days (D) after transfection and cross sections were double-stained with H&E and von Kossa. (A) Dotted distribution of thin regenerating fibers can be seen (arrows). (B) Original structure of muscle is almost preserved, except for slight fibrosis and small-cell infiltrations in the extracellular space (arrows), probably at the injected site. No bone formation can be observed in (A) and (B). (C) Endochondral ossification occurs in almost the whole sectional areas of the muscle. A cartilage zone (an arrow) is seen adjacent to rather delicate trabeculae of mineralized bone, which exhibit von Kossa–stained $CaPO_4$ deposits (asterisks). (D) A thick cortex (asterisk) with a vast marrow sinus (arrowheads) occupying the whole muscle is formed at day 28 after *BMP-2* gene transfer and grafting. The marrow sinus is occupied by colonies of hematopoietic cells and adipocyte-like cells, resembling the yellow pulp of human bone marrow. Bar, 100 μm.

BMPR-IA-positive cells were up-regulated after a transient decrease following muscle grafting (Figs. 7D and 7E). The total number of nuclei did not show a significant change throughout the experimental period (Fig. 8A). The percentage of BMPR-IA-positive cells at 24 h after muscle grafting was significantly smaller than at days 3, 7, and 14. It reached the maximum on day 7 and then decreased (Fig. 8B). The real number of BMPR-IA-positive cells followed almost the same course as the percentage (data not shown).

Similar analysis was made on the *BMP-2* gene-transferred muscles and as a result, the overexpression of *BMP-2* transgene did not significantly affect the receptor up-regulation (data not shown). No staining of BMPR-1B was observed in any muscle specimens ($n = 3$ at each time point; data not shown).

DISCUSSION

In the present study, massive heterotopic ossification was induced throughout almost the whole lengths of the grafted muscles transfected with a replication-deficient adenovirus carrying the *BMP-2* gene, as shown in the BMP-graft group. Heterotopic bone formation was successfully induced by exogenous BMP-2 in a number of previous studies, in which different kinds of delivery carriers were impregnated with BMPs and then implanted intramuscularly or subcutaneously.[11–14] Their bone formations, however, were rather small and the ossification induced in our model far exceeded them in terms of its magnitude. Also, the lack of bone formation in the BMP-muscle group in our study was in sharp contrast with these studies. It has been reported that, in the case of graft-induced muscle degeneration, a number of cells such as migratory phagocytes and proliferating satellite cells are recruited.[20] We speculate that tissue damage and chemoattraction might have been evoked by implantation of BMP carriers in the past studies, resulting in the recruitment of a variety of cells associated with tissue restoration and the immune response, but not in the BMP-muscle group in our study. It is also possible, however, that the failed bone formation in the BMP-muscle group may be partly the result of the smaller expression of the transgene in ungrafted muscles compared with that in grafted muscles.

In the present study, ossification of the rat soleus muscle in the BMP-graft group was preserved for at least 1 year (Fig. 5F). Probably the BMP-2 action is required to trigger the bone formation cascade in the early phase. Also, graft-induced muscle regeneration was strongly inhibited when transfected with the adenovirus-carrying *BMP-2* gene. The result indicates that BMP-2 has an inhibitory action on the myogenic differentiation of precursor cells in vivo, which is consistent with the past in vitro studies that demonstrated that BMP-2 inhibits the myogenic differentiation of cultured cells.[37,38]

It is noteworthy that BMPR-IA-positive cells were up-regulated after a transient decrease following muscle grafting. First of all, this transient decrease might be the result of ischemic necrosis of the satellite cells and/or perivascular mesenchymal cells. In the ungrafted muscles, preexisting BMPR-IA-positive cells did not efficiently respond to BMP-2 expression in terms of bone formation. One explanation for this is that their cell number was too small or the BMP-2 expression was too low to induce bone formation in skeletal muscle. Another possibility is that preexisting BMPR-IA-positive cells and those that were newly recruited by the muscle grafting were phenotypically distinct and thus showed different responses to BMP-2. It is also possible that unknown factors associated with the muscle grafting might have been involved in the osteogenic action of BMP-2. On the other hand, in the grafted muscle, the up-regulation of BMPR-IA-positive cells presumably played a critical role in the BMP-2-induced ossification. The striking contrast in bone formation between the BMP-muscle and BMP-graft groups in our study underscores the significance of injury-induced BMP-receptor expression in the experimental BMP-induced bone formation. The origin of osteoprogenitor cells in our animal model, as well as the possibility that those BMPR-IA-positive cells up-regulated after grafting could serve as osteoprogenitor cells, remains an intriguing question that should be investigated.

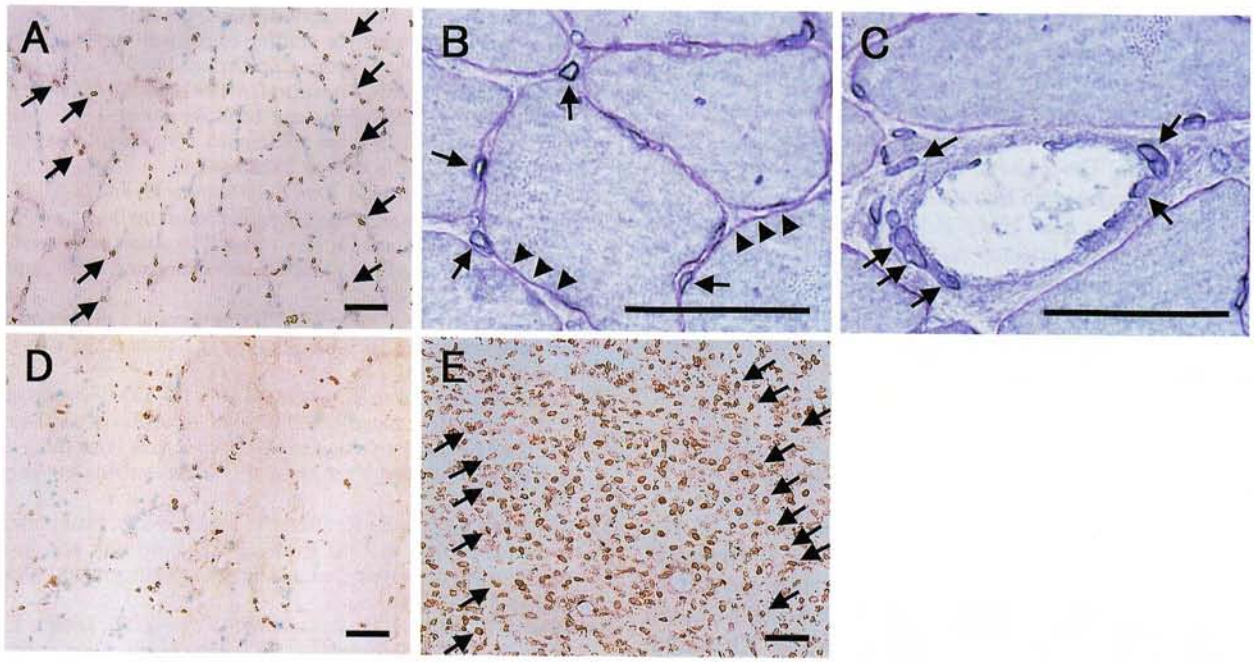

FIG. 7. Immunolocalization of BMP receptor type IA (BMPR-IA) in rat soleus muscles before and after orthotopic grafting. Ungrafted muscles (A–C) and orthotopically grafted muscles, 24 h (D) and 7 days (E) after grafting, were harvested. No virus transfection was performed. Cross sections were immunostained with anti-BMPR-IA (A, D, E) and double-stained with anti-BMPR-IA and Dystrophin (B, C). (A) Small cells located along myofiber endomysium (arrows), suggestive of satellite cells, predominantly expressed BMPR-IA. (B and C) BMPR-IA-positive cells were labeled with gray (arrows) and Dystrophin on myofiber membranes with purple (arrowheads). BMPR-IA-positive cells in (B) were located in the endomysium outside the plasma membranes, strongly suggesting that they were satellite cells. Those cells were also located in the connective tissue around the intramuscular small vessels. (D and E) BMPR-IA-positive cells transiently decreased in number at 24 h (D), and then remarkably increased at day 7 (E). Note that only infiltrating small cells (arrows), but not regenerating fibers, express BMPR-IA at day 7 (E). Bar, 50 μm.

The present study showed not only that skeletal muscle can function as a delivery carrier for recombinant BMP-2 protein but also that it could even serve as a bone substitute. Clinically, recent advances in microsurgical techniques have made possible the autogenous transplantation of skeletal muscle to a distant diseased site.[39] Thus, bone transformation of a grafted skeletal muscle would appear to be very useful for treating impaired regional bone formation, including delayed or nonbone fracture union, congenital pseudoarthrosis occurring either alone or in association with von Recklinghausen's disease, and segmental bone defects after trauma, osteomyelitis, or tumor resection. However, for the application of this system of adenovirus-mediated *BMP-2* gene transfer for human therapies, inducing the degeneration/regeneration of the grafted muscle would pose another problem, because no clinical condition has ever been known to show similar features as those in the BMP-graft group in this study. For example, free nonvascularized grafting of a human skeletal muscle would not result in regeneration but in the total necrosis of the muscle, probably because angiogenetic vascular ingrowth into grafted muscle is not as vigorous in human tissues as in rodent tissues. Moreover, no ischemic degeneration is supposed to occur in vascularized muscle flap transplantation. An alternative way to induce muscle regeneration in human tissue as it occurred

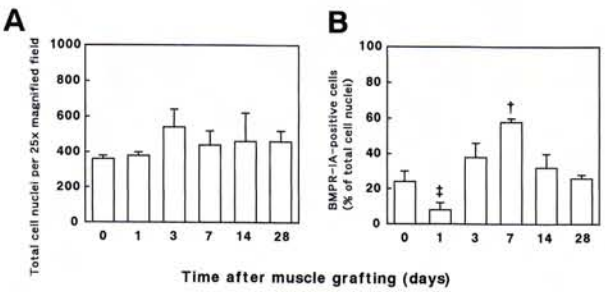

FIG. 8. Time course of the number of total cell nuclei and the percentage of BMPR-IA-positive cells in the rat soleus muscles after orthotopic grafting. No transfection was performed. Cross sections were immunostained with anti-BMPR-IA and counterstained with methyl green to determine the total number of cell nuclei as an internal control. The cells or nuclei numbers were determined on each double-stained section and the results are expressed as an average number of total cell nuclei per ×25 magnified fields (A) and the percentage of BMPR-IA-positive cells to total cell nuclei (B) for each specimen. Values are means ± SD for three specimens. $^{\dagger}p < 0.05$ versus all other days and $^{\ddagger}p < 0.05$ versus days 3, 7, and 14.

in the grafted muscles in this study should be sought. However, considering the multitude of muscle graft donor sites[40] and the comparatively small donor site morbidity in muscle harvest, the clinical application of our system of gene therapy would be promising, thus deserving further investigation. The present study is only the first step in genetically engineered tissue creation.

In conclusion, our study indicates that the strategy of adenovirus-mediated gene transfer as a delivery system for BMP-2 can be effective for in vivo osteoinduction when the target muscle is grafted, although its inefficiency without muscle grafting poses some difficulty in terms of possible in vivo application.

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