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Gene Expression Analysis of Ectopic Bone Formation Induced by Electroporatic Gene Transfer of BMP4

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ABSTRACT

Implantation of bone morphogenetic protein (BMP) using a carrier or by BMP gene transfer into rodent muscle can induce bone formation. Implanted BMP becomes bioactive immediately after implantation. In BMP gene transfer, there is a time-lag between the secretion of gene products and bone formation. We analyzed the gene expression of chondrogenic and osteogenic specific markers in the process of ectopic bone formation by using semi-quantitative RT-PCR. A plasmid vector containing mouse BMP4 gene (pCAGGS-BMP4) was transferred into the gastrocnemius muscles of mice using electroporation. Histological examination revealed the process of endochondral bone formation in the pCAGGS-BMP4 transferred muscles. As chondrogenic markers, aggrecan gene maximal expression was detected on day 7 and decreased by day 14, and for collagen X the gene maximal expression was on day 10. As osteogenic markers, osteocalcin (*OCN*), bone sialoprotein (*BSP)* and osteopontin (*OPN*) gene expressions were clearly detected from day 10 and then increased by day 14. In conclusion, the present study proved that ectopic bone formation by BMP4 gene transfer into the muscle induced endochondral ossification that corresponded well with that to that by implantation of demineralized bone matrix.

INTRODUCTION

Bone morphogenetic protein (BMP) induces or stimulates bone formation. Some isoforms of BMPs (BMP2, 4, 6, and 7) differentiate mesenchymal cells into osteoblastic linage cells (1-3). In classical experiments using BMPs, a carrier is necessary for bone induction because BMPs have a short half-life *in vivo* (4). Appropriate carrier implantation allows gradual BMPs release and works as a scaffold for cells (5).

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Gene therapy technique is thought to be a potent option for utilizing BMPs. When a BMP coding gene is introduced into cells, the cells continuously produce and secrete BMP. Adenoviral transfer of a BMP coding gene into rodent muscle showed abundant ectopic bone formation in immunodeficient animals without a carrier (6). We previously reported that electroporatic transfer of a plasmid DNA containing mouse *BMP*-4 induced bone in immunocompetent mice (7).

It is difficult to quantify the amount of protein produced by gene-transferred cells in an *in vivo* animal model. The pharmacokinetics of BMPs might be different between protein and gene based experiments. BMPs existing in demineralized bone matrix or put in a carrier are abundant at the time of implantation *in vivo* and decrease gradually. On the other hand, BMP expression, theoretically, increases after gene transfer, continues for weeks and then decreases gradually.

The molecular and cellular events involved in endochondral ossification after implantation of demineralized bone matrix were described in detail by Reddi (8). By day 3, mesenchymal and inflammatory cells had appeared around the implant. Chondroblasts appeared by day 5 and chondrocytes were maximal on day 7. The hypertrophic cartilage matrix began to undergo calcification by day 9. Basophilic osteogenic precursors and osteoblasts appeared on day 10. Bone formation was confirmed on days 12 to 18. In this cascade of endochondral ossification, each differentiation step of osteogenic and chondrogenic cells was analyzed by using the following markers: osteocalcin (9), osteopontin (9), bone sialoprotein (10, 11), aggrecan (12, 13), and type X collagen (14). However, these markers of gene expression in this cascade were not assessed after BMP gene transfer but in protein and carrier models (15-17). To the best of our knowledge, this paper is the first to confirm the temporal gene expressions of osteogenic and chondrogenic markers in ectopic bone formation caused by the introduction of BMP4 gene into the muscle.

MATERIALS AND METHODS

Mice

Male C57BL/6J mice were purchased from Clea Japan, Inc. Electroporation was performed on 8-week-old mice. Mice were maintained under specific pathogen-free conditions at the Institute for Animal Experimentation, Tohoku University School of Medicine. The Institutional Animal Care and Use Committee approved all the procedures used in this study.

Plasmid DNA

The 1.6kb mouse BMP-4 cDNA was kindly provided by Brigid L. M. Hogan (18). It was inserted into multiple cloning sites of a pCAGGS expression vector (19) (pCAGGS-BMP4). pCAGGS-GFP, a GFP-containing plasmid, was used as the control. Both plasmids were dissolved in distilled water at 2.0μg/μl. 100 microgrms of each was injected into the animals.

In Vivo Electroporation
Fifty microliters of 0.5% bupivacaine were injected into the left gastrocnemius as a pretreatment (20). In vivo electroporation was performed as previously described (pulse settings: 100V, 50ms, 6pulses, 1Hz) (7, 20). Pulses were applied through percutaneously inserted electrodes (0.4mm diameter: Unique Medical Imada, Natori, Japan) just after injection of pCAGGS-BMP4 or pCAGGS-GFP (50μl each) into the pretreatment site. Mice were euthanized by cervical dislocation 3, 7, 10, or 14 days after electroporation.

Soft X-ray assessment
All gastrocnemius muscles were excised after sacrifice and underwent soft X-ray examination at 20.0kV and 2.0mA, for 10 sec (SRO-iM50, Sofron, Tokyo) using X-Omat TL film (Kodak).

Histology and Immunohistochemistry
Frozen non-decalcified sections (thickness 10μm) of the specimens were made with a cryostat (Bright, UK) after the soft X-ray photography. Slides were stained with hematoxylin-eosin, alcian blue or von-Kossa. BMP4 expression was detected immunohistochemically by rabbit polyclonal anti-BMP-4 antibody (1:100 dilution, overnight at 4°C; Santa Cruz Biotechnology, Santa Cruz, CA). HRP labeled goat anti-rabbit antibody was used as the secondary antibody (1:100, 2h at room temperature; Dako Cytomation).

RT-PCR analysis
A muscular portion of the gastrocnemius with pCAGGS-BMP4 (n=6) or pCAGGS-GFP (n=6), 10 mm in length containing the center of the electroporated site was dissected (0.12g).

Total RNA was isolated from the dissected muscles with the RNeasy Fibrous Tissue Mini Kit (QIAGEN, Germany) and reverse transcription-PCR was done with a TaKaRa One Step RNA PCR Kit (Takara, Japan) following the manufacturer's instructions. The examined marker genes and their primers (in 5' to 3' direction) were as follows:

Transgene marker:
mouse BMP4 (accession number: X56848)
Fwd. CCCAGAGAATGAGGTGATCTCC
Rev. TGGCAGTAGAAGGCCTGGTAG

Chondrogenic markers:
procollagen type Xalpha 1 (*COL10* accession number: Z21610)
Fwd. GCCAGGTCTCAATGGTCCTA
Rev. GCACCTACTGCTGGGTAAGC
aggrecan (accession number: L07049)
Fwd. CAGGTTTCCCCACTGTGTCT
Rev. ACTCCAGACCCTGGGAAGTT

Osteogenic markers:
osteocalcin (*OCN* acession number: X04142)
Fwd. CTTGGTGCACACCTAGCAGA
Rev. ACCTTATTGCCCTCCTGCTT
osteopontin (*OPN* accession number: AF515708)
Fwd. TCTGATGAGACCGTCACT
Rev. TCTCCTGGCTCTCTTTGGAA
bone sialoprotein (*BSP* accession number: L20232)
Fwd. AAAGTGAAGGAAAGCGACGA
Rev. GTTCCTTCTGCACCTGCTTC

Internal control:
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH* accession number: M32599)
Fwd. TGTTTGTGATGGGTGTGAA
Rev. ATGGGAGTTGCTGTTGAA

Total RNA (1μg) was incubated for 30 minutes at 50˚C in a total volume of 50μl and then for 2 min at 94˚C, followed by 25 cycles for 30 seconds at 94˚C, for 30 seconds at 60˚C and then for 30seconds at 72˚C. The PCR products were analyzed on 2% agarose gels and visualized with ethidium bromide. The density of each band was quantified using Scion Image software (http://www.scioncorp.com). We determined the relative gene expression by dividing the densitometry value of the mRNA RT-PCR product by that of the GAPDH product.

Statistical analysis
All measurements were performed in triplicate for each specimen and the mean value was used for statistical analysis. Results were presented as mean ± standard deviation. The significance of differences between control and pCAGGS-BMP4 electroporated muscles was determined using Mann-Whitney U test. Values less than P=0.01 were considered significant.

RESULTS

X-ray assessment
A radio-opaque area was observed in the gastrocnemius of all animals electroporated with pCAGGS-BMP4 after 14days, but not in those electroporated with pCAGGS-GFP.

Histological findings
Mesenchymal cell infiltration was observed 3 days after BMP4 electroporation (Figure. 1A). On day 7, the extracellular matrix was found to be stained clearly with alcian blue (Figure. 1B, C). On day 10, when hypertrophic chondrocytes and cartilagenous matrix appeared, the intensity of alcian blue staining decreased (Figure. 1D, E). The extracellular matrix was not revealed by von Kossa staining. On day 14, calcified bone matrix was detected with von Kossa staining (Figure. 1F).

After electroporation of control pCAGGS-GFP, mesenchymal cell infiltration was also observed on day 3. Calcium deposits between the muscle bundles were sporadically observed on day 7. No cartilage or bone matrix were found at any time point (data not shown). Immunohistologic analysis revealed that BMP4 was expressed in muscle fibers on day 3 (Figure. 2A), and the intensity of BMP4 expression decreased with time (Figure. 2B, C, D).

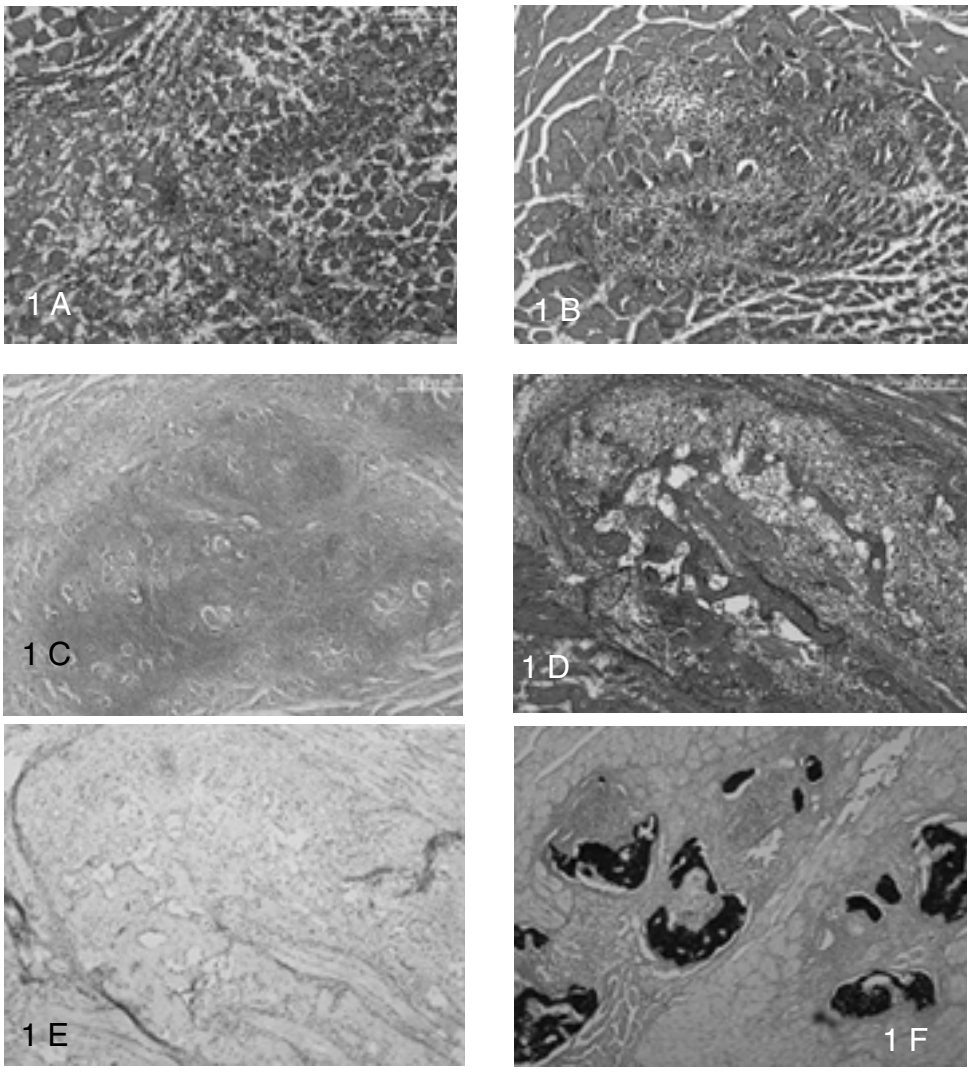

Fig 1. Axial sections of pCAGGS-BMP4 electroporated muscles stained by hematoxylin-eosin (A, B, D), alcian-blue (C, E) and von Kossa (F). Infiltrations of mesenchymal cells were found between muscle fibers on day 3 (A). Extracellular matrices stained with alcian blue were identified on day 7 (B, C). Hypertrophic chondrocytes and cartilage matrices were observed on day 10 (D, E). Calcified bone matrices stained with von Kossa were identified on day 14. Bone marrow-like cells were found in calcified matrices (F).

Semi-quantitative RT-PCR

BMP4 mRNA expression was clearly detected 3 days after BMP4 electroporation. It gradually decreased and was hardly detected on day 14. No BMP4 mRNA expression was detected in control (Figure. 2E). Aggrecan mRNA expression was detected in BMP4 electroporated muscles from day 7, and decreased by day 14. No aggrecan mRNA expression was detected in control. *COL10* mRNA expression in

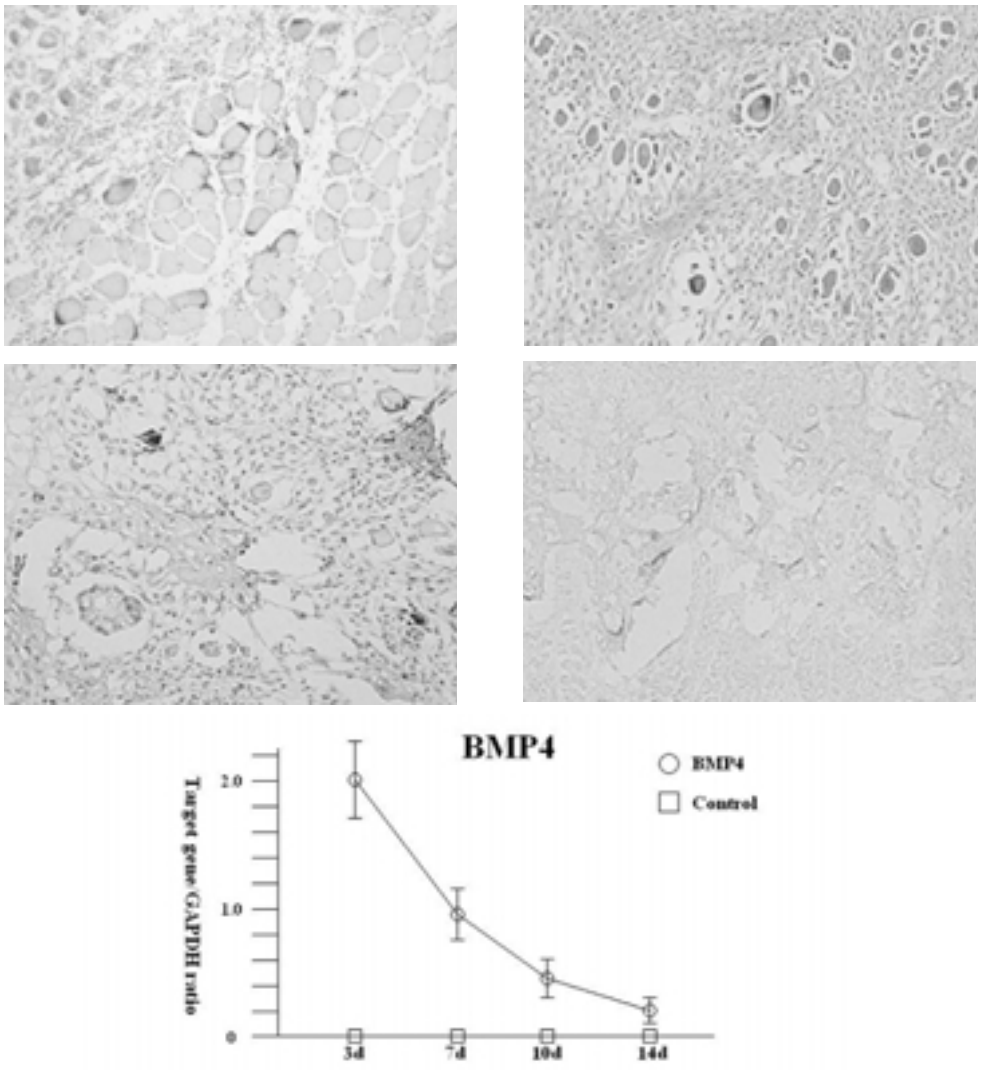

Fig 2. Localization of BMP4 expression analyzed by *in situ* immunohistochemistry at 3 (A), 7 (B), 10 (C) and 14 days (D) after electroporation. BMP4 was highly expressed in muscle fibers surrounding infiltrated mesenchymal cells on day 3 (A). The intensity of BMP4 expression and the number of BMP4 positive cells decreased with time. Reverse transcription-polymerase chain reaction (RT-PCR) showed intense mouse BMP4 mRNA expression in pCAGGS-BMP4 electroporated muscles on day 3 and its expression decreased by day 14. BMP4 mRNA expression was not detected in pCAGGS-GFP (control) electroporated muscles (E).

BMP4 electrtoporated muscles was significantly higher than in the control on day 10 and 14 (Figure 3A,B). *OCN* and *BSP* mRNA expressions were detected in BMP4 electroporated muscles on day 7. They were significantly elevated on day 10 and increased on day 14. No *OCN* and *BSP* mRNA expressions were detected in control. *OPN* mRNA expression was high on day 3, not detected on day 7, detected again on day 10 and increased on day 14 in BMP4 eletroporated muscles. In control, *OPN* mRNA expression was not detected after day 7 (Figure. 4A, B, C).

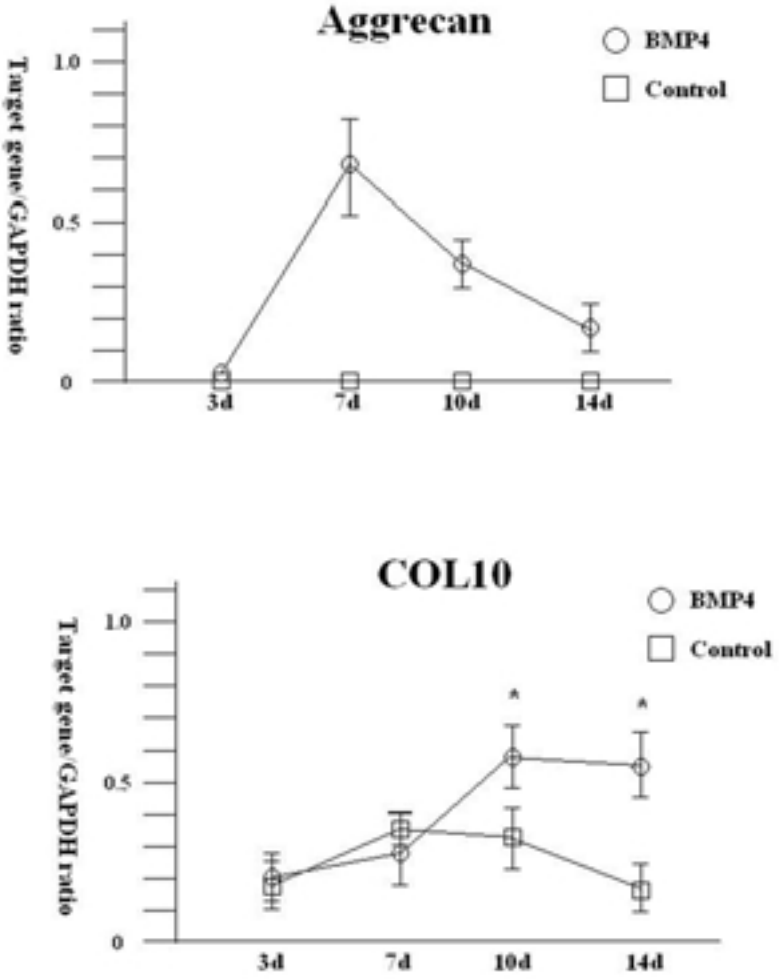

Fig 3. Time course of chondrogenic marker genes expression. Aggrecan mRNA expression was initially detected on day 7, and decreased by day 14, whereas it was not detected in the control group throughout this entire period (A). Procollagen type X alpha 1 mRNA expression was significant higher than in the control group on day 10 and 14 (B). The I-bars represent the standard deviation and the asterisk (*) indicates significant differences in mRNA expression at each time point ($p < 0.01$).

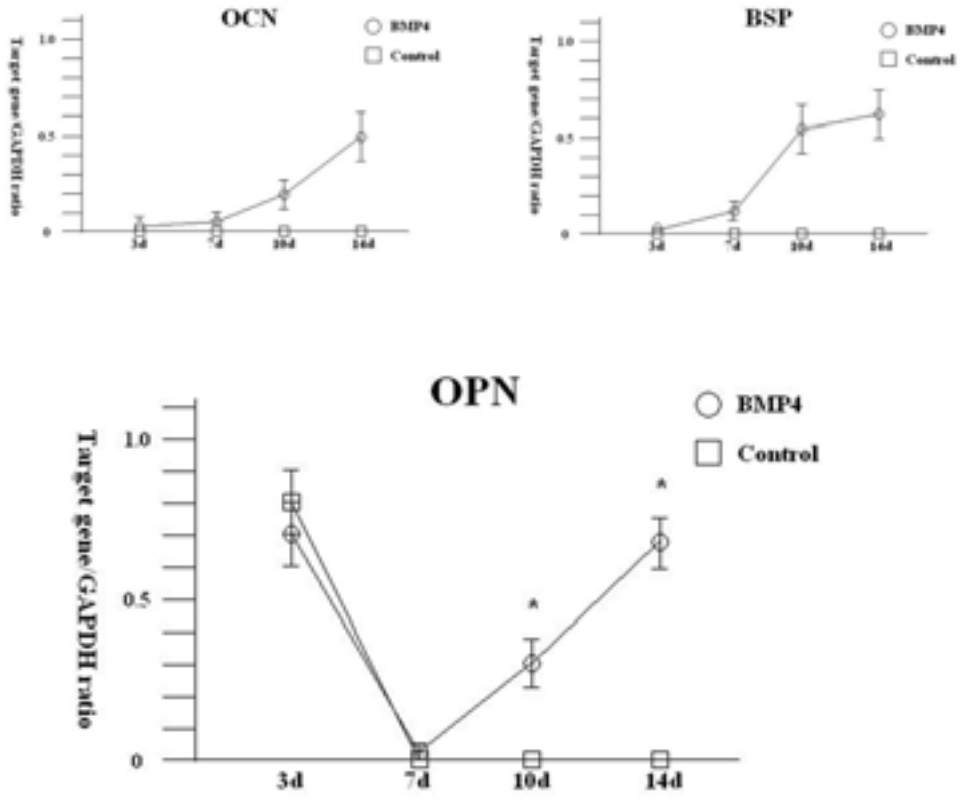

Fig 4. Time course of osteogenic marker gene expression. Osteocalcin and bone sialoprotein mRNA expressions were detected in the pCAGGS-BMP4 group on day 7, were significantly elevated on day 10 and further increased on day 14. Osteocalcin and Bone sialoprotein mRNA expressions were not detected in the control group (A,B). Osteopontin mRNA expression was high in both groups on day 3. Its expression in pCAGGS-BMP4 electroporated muscles was decreased on day 7 and gradually increased from day 10 and to day 14. Osteopontin mRNA expression in control group was not detectable after day 7 (C). The I-bars represent the standard deviation and the asterisk (*) indicates significant differences in mRNA expression at each time point ($p < 0.01$).

DISCUSSION

Gene transfer using electroporation is safe and inexpensive. In 2002, the present authors reported bone induction by using electroporatic transfer of BMP4, and the rate of bone formation in BALB/cA mice was 67% at 14 days after electroporation (7). It is noteworthy that the present experiment demonstrated 100 % bone formation at 14

days. In this study, we could induce bone formation in all BMP4 transferred C57BL/6J mice. There are three possible reasons why the bone formation rate improved. The first is that the C57BL/6J strain, is reportedly a better responder for BMPs than the BALB/cA strain (21). The second reason may be that the pCAGGS expression vector containing the CAG promoter has higher activity in muscle (19). The third is that pretreatment with bupivacaine, which induces muscle necrosis, may have enhanced the efficiency of gene transfer by direct intramuscular plasmid injection (22-24). Additionally, the muscle regeneration process after the pretreatment could provide abundant mesenchymal cells, which are essential for ectopic bone formation (25). However, at 14 days, bone induction was also observed in all mice even without Bupivacaine pretreatment (data not shown), which lead us to believe that the strain of the mouse and the change of the expression plasmid contributed to stable bone formation.

It is well known that transplantation of BMP protein or gene transfer of BMP into the muscles induce bone formation. However, there have been no reports describing in detail the cell differentiation in the process of bone formation. It is thought to be difficult to clearly distinguish each stage of differentiation of osteoblastic or chondroblastic cells under microscopic observation alone. However, we can recognize differentiation stages by serially examining the gene expression changes of osteogenic and chondrogenic markers.

Aggrecan is an extracellular matrix of cartilage and its gene is reported to be expressed in proliferating chondrocytes (13). *COL10* gene is reported to be expressed specifically in hypertrophic chondrocytes (26). Aggrecan and *COL10* mRNA expressions and histological examination in our experiment indicated that proliferating chondrocytes appeared on day 7 and had differentiated into hypertrophic chondrocytes by day 10.

The gene expressions of *OCN* and *OPN* were reported to correlate with the appearance of mature osteoblasts (27,28). BSP is extracellular matrix protein produced at the phase of osteoblastic differentiation (29). The OCN and *OPN* mRNAs expressions in our experiment indicated that differentiation into osteoblasts and bone matrix formation had started by day 10. *OPN* mRNA expression was strongly detected on day 3 in both BMP4 electroporated and control muscles. According to a recent report, *OPN* may serve as a molecule that promotes macrophage binding to nextrotic fibers and may be an impotant mediator in the early phase of muscle regeneration (30). The *OPN* mRNA expression on day 3, therefore, does not reflect the appearance of osteoblasts but the activation of these inflammatory cells.

In conclusion, examining changes of osteoblastic and chondroblastic gene expression enabled us to understand the differentiation stages of the cells resulting from BPM4 gene transfer. The present study proved that ectopic bone formation by BMP4 gene transfer into the muscle induced endochondral ossification that corresponded well to that by implantation of demineralized bone matrix.

REFERENCES

1. Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, Rosen V, Wozney JM, Fujisawa-Sehara A, Suda T (1994) Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol 127: 1755-1766.
2. Ebisawa T, Tada K, Kitajima I, Tojo K, Sampath TK, Kawabata M, Miyazono K, Imamura T (1999) Characterization of bone morphogenetic protein-6 signaling pathways in osteoblast differentiation. J Cell Sci 112: 3519-3527.
3. Asahina I, Sampath TK, Nishimura I, Hauschka PV (1993) Human osteogenic protein-1 induces both chondroblastic and osteoblastic differentiation of osteoprogenitor cells derived from newborn rat calvaria. J Cell Biol 123: 921-933.
4. Wakefield LM, Winokur TS, Hollands RS, Christopherson K, Levinson AD, Sporn MB: (1990) Recombinant latent transforming growth factor beta 1 has a longer plasma half-life in rats than active transforming growth factor beta 1, and a different tissue distribution. J Clin Invest 86: 1976-1984.
5. Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA (1988) Novel regulators of bone formation: molecular clones and activities. Science 242: 1528-1534.
6. Musgrave DS, Bosch P, Ghivizzani S, Robbins PD, Evans CH, Huard J (1999) Adenovirus-mediated direct gene therapy with bone morphogenetic protein-2 produces bone. Bone 24: 541-547.
7. Kishimoto KN, Watanabe Y, Nakamura H, Kokubun S (2002) Ectopic bone formation by electroporatic transfer of bone morphogenetic protein-4 gene. Bone 31: 340-347.
8. Reddi AH (2001) Bone morphogenetic proteins: from basic science to clinical applications. J Bone Joint Surg Am 83: 1-6.
9. Stein GS, Lian JB (1993) Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. Endocr Rev 14: 424-442.
10. Bianco P, Fisher LW, Young MF, Termine JD, Robey PG (1991) Expression of bone sialoprotein (BSP) in developing human tissues. Calcif Tissue Int 49: 421-426.
11. Chen J, Shapiro HS, Sodek J (1992) Development expression of bone sialoprotein mRNA in rat mineralized connective tissues. J Bone Miner Res 7: 987-997.
12. Doege KJ, Sasaki M, Kimura T, Yamada Y (1991) Complete coding sequence and deduced primary structure of the human cartilage large aggregating proteoglycan, aggrecan. Human-specific repeats, and additional alternatively spliced forms. J Biol Chem 266: 894-902.
13. Walcz E, Deak F, Erhardt P, Coulter SN, Fulop C, Horvath P, Doege KJ, Glant TT (1994) Complete coding sequence, deduced primary structure, chromosomal localization, and structural analysis of murine aggrecan. Genomics 22: 364-371.
14. Gerstenfeld LC, Cullinane DM, Barnes GL, Graves DT, Einhorn TA (2003) Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. J Cell Biochem 88: 873-884.
15. Clancy BM, Johnson JD, Lambert AJ, Rezvankhah S, Wong A, Resmini C, Feldman JL, Leppanen S, Pittman DD (2003) A gene expression profile for endochondral bone formation: oligonucleotide microarrays establish novel connections between known genes and BMP-2-induced bone formation in mouse quadriceps. Bone 3: 46-63.
16. Kubota K, Iseki S, Kuroda S, Oida S, Iimura T, Duarte WR, Ohya K, Ishikawa I, Kasugai S (2002) Synergistic effect of fibroblast growth factor-4 in ectopic bone formation induced by bone morphogenetic protein-2. Bone 31: 465-471.

17. Takita H, Vehof JW, Jansen JA, Yamamoto M, Tabata Y, Tamura M, Kuboki Y (2004) Carrier dependent cell differentiation of bone morphogenetic protein-2 induced osteogenesis and chondrogenesis during the early implantation stage in rats. J Biomed Mater Res A 71: 181-189.
18. Jones CM, Lyons KM, Hogan BL. (1991) Involvement of Bone Morphogenetic Protein-4 (BMP-4) and Vgr-1 in morphogenesis and neurogenesis in the mouse. Development 111: 531-542.
19. Niwa H, Yamamura K, Miyazaki J (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene Dec 108 : 193-199.
20. Aihara H, Miyazaki J (1988) Gene transfer into muscle by electroporation in vivo. Nat Biotechnol 16: 867-870, 1998
21. Marusic A, Katavic V, Grcevic D, Lukic IK (1999) Genetic variability of new bone induction in mice. Bone 25: 25-32.
22. Davis HL, Demeneix BA, Quantin B, Coulombe J, Whalen RG (1993) Plasmid DNA is superior to viral vectors for direct gene transfer into adult mouse skeletal muscle. Hum Gene Ther 4: 733-740.
23. Vitadello M, Schiaffino MV, Picard A, Scarpa M, Schiaffino S (1994) Gene transfer in regenerating muscle. Hum Gene Ther 5: 11-18.
24. Wells DJ. (1993) Improved gene transfer by direct plasmid injection associated with regeneration in mouse skeletal muscle. FEBS Lett 332: 179-182.
25. Gonda K, Nakaoka T, Yoshimura K, Otawara-Hamamoto Y, Harrii K (2000) Heterotopic ossification of degenerating rat skeletal muscle induced by adenovirus-mediated transfer of bone morphogenetic protein-2 gene. J Bone Miner Res15: 1056-1065.
26. Kong RY, Kwan KM, Lau ET, Thomas JT, Boot-Handford RP, Grant ME, Cheah KS (1993) Intron-exon structure, alternative use of promoter and expression of the mouse collagen X gene, Col10a-1. Eur J Biochem 213: 99-111.
27. Leali D, Dell'Era P, Stabile H, Sennino B, Chambers AF, Naldini A, Sozzani S, Nico B, Ribatti D, Presta M (2003) Osteopontin (Eta-1) and fibroblast growth factor-2 cross-talk in angiogenesis. J Immunol. 171: 1085-1093.
28. Lee K, Deeds JD, Chiba S, Un-No M, Bond AT, Segre GV (1994) Parathyroid hormone induces sequential c-fos expression in bone cells in vivo: in situ localization of its receptor and c-fos messenger ribonucleic acids. Endocrinology 134: 441-450.
29. Young MF, Ibaraki K, Kerr JM, Lyu MS, Kozak CA (1994) Murine bone sialoprotein (BSP): cDNA cloning, mRNA expression, and genetic mapping. Mamm Genome 5: 108-111.
30. Hirata A, Masuda S, Tamura T, Kai K, Ojima K, Fukase A, Motoyoshi K, Kamakura K, Miyagoe-Suzuki Y, Takeda S (2003) Expression profiling of cytokines and related genes in regenerating skeletal muscle after cardiotoxin injection: a role for osteopontin. Am J Pathol. 163: 203-215.

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