

Immune response and effect of adenovirus-mediated human BMP-2 gene transfer on the repair of segmental tibial bone defects in goats

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Background Tissue-engineered bone may be used for filling bone defects. There are, however, no reports on this technique used in large animals.

Methods We evaluated the effectiveness of, and immune response in repairing diaphyseal bone defects by gene transfer using bone morphogenetic proteins (BMPs). We used adenovirus-mediated human BMP-2 (Adv-hBMP-2) gene-transduced bone marrow stromal cells (BMSCs) to repair 2.1-cm segmental tibial bone defects in goats (group I, n = 7). An Adv-βgal-transduced BMSC group (group II, n = 5), a non-transduced BMSC group (group III, n = 5), and an untreated group (group IV, n = 2) were used as controls. Self-secreted extracellular matrix was used as cellular carrier.

Results Radiographic and histomorphometric examination demonstrated more callus in the bone defects of group I compared to other groups. Week 24 after implantation, the defect healing rates of groups I, II, III, and IV were 6/7, 1/5, 2/5, and 0/2, respectively. The maximum compressive strength of new tissue in the bone defects of group I was higher than those of groups II and III. Temporary cellular and persistent humoral immune responses against adenovirus were detected after hBMP-2 gene transfer.

Interpretation We found that Adv-hBMP-2 gene-transduced BMSCs had superior osteoinductivity in the repair of tibial bone defects in goats, but it could cause temporary cellular and persistent humoral immune responses against adenovirus.

Tissue-engineered bone is a new approach in repairing bone defects. An ideal tissue-engineered bone substitute should possess 3 elements: osteoprogenitor cells, osteoinductive factors, and an osteoconductive scaffold (Joseph et al. 1999). Bone morphogenetic proteins (BMPs) are a group of acidic polypeptides that play a key role in the formation and repair of bone (Reddi 2001). Among them, BMP-2 is one of the strongest members (Riley et al. 1996). Recently, gene transfer of BMP-2, 4, 7, and 9 was successfully used to achieve in vivo osteoinduction in athymic mice (Lou et al. 1999, Krebsbach et al. 2000, Musgrave et al. 2000, Okubo et al. 2001, Abe et al. 2002, Chen et al. 2003, Luk et al. 2003, Zhu et al. 2003), repair of radial, femoral, periodontal and cranial bone defects in mice, rats, and rabbits (Lieberman et al. 1999, Baltzer et al. 2000, Lee et al. 2001, 2002, Xu et al. 2002, Jin et al. 2003, Tsuchida et al. 2003, Chang et al. 2003), spinal fusion in rats, rabbits, and pigs (Riew et al. 1998, 2003, Dumont et al. 2002, Wang et al. 2003), and enhancement of tendon-bone integration (Martinek et al. 2002). There has been no report, however, of BMP gene transfer inducing the repair of segmental diaphyseal defects in large animals.

Two kinds of cell carriers have been used for bone tissue engineering. Osteoconductive carriers include hydroxyapatite (Kon et al. 2000), coral (Petite et al. 2000) and so on (Xu et al. 2003); poorly osteoconductive carriers include alginate, gelatin (Lee

et al. 2001), extracellular matrix (ECM) (Lieberman et al. 1999), and collagen gel or sponge (Riew et al. 1998, Jin et al. 2003, Tsuchida et al. 2003). No studies have made use of extracellular matrix (ECM) synthesized by implanted cells themselves as the carrier in bone tissue engineering.

There are several choices of vector for BMP-2 gene transfer such as plasmids, liposomes, retrovirus, adenovirus, and adeno-associated virus (AAV). The replication-deficient adenoviral vector has many superior characters including wide host range, high titer of amplification, ability to infect quiescent cells, and a lower potential for oncogenesis and teratogenesis than with retroviral vectors because there is no insertion of DNA into the host genome (Tauber and Dobner 2001). In 1999, Jesse, a patient with ornithine transcarbamylase (OTC) deficiency, died of multiple organ failure after his hepatic artery was injected with genetically altered adenovirus (Adv), so increasing attention has been paid to the effect of host immune responses against adenovirus-mediated gene therapy (Harvey et al. 1999, Stein et al. 2000, Rahman et al. 2001, Nikitina et al. 2002). It has been reported that intradermal administration of an adenoviral vector to humans induced local cellular responses and systemic Adv5-specific lymphocyte proliferation (Harvey et al. 1999). It has also been reported that the level of neutralizing anti-adenovirus antibody is moderately elevated in mice that have received adenovirus-mediated gene therapy (Nikitina et al. 2002). However, the duration of the immune response was not very clear. No research on immune responses after BMP gene transfer has been reported, especially using the *ex vivo* method of gene transfer combined with tissue engineering techniques in large animals.

Before this study, we succeeded in inducing hBMP-2 secretion, osteoblast differentiation, and ectopic bone formation in athymic mice from adenovirus-mediated human BMP-2 (Adv-hBMP-2) gene-transduced bone marrow stromal cells (BMSCs). We have also achieved repair of rat femoral and rabbit radial bone defects using hBMP-2 gene transfer (Xu et al. 2002, 2003, Zhu et al. 2003).

In the present study, in order to clarify whether Adv-hBMP-2 gene transfer can repair segmental diaphyseal bone defects in large animals and clarify

the duration and intensity of cellular and humoral immune responses, we used Adv-hBMP-2 transduced goat BMSCs to repair segmental tibial bone defects in goats. These secreted ECM and formed membranous cellular masses. The host cellular and humoral immune responses against adenovirus were assayed before and after implantation.

Material and methods

Culture of BMSCs and gene transfer

8 mL bone marrow was drawn from iliac bone of each goat and cultured in minimum essential medium alpha medium (α -MEM) (11900; Gibco-BRL, Gaithersburg, MD) with 10% fetal bovine serum (FBS) (SH30088.03; Hyclone, Logan, UT). BMSCs, a spectrum of different cells, can adhere to the surface of culture dishes (whereas hemopoietic cells cannot), so the adherent cells were isolated from bone marrow and used at passage 2 for experiments (Riew et al. 1998, Xu et al. 2002). Flow cytometry showed the percentage of cells positive for CD44, CD90, CD71, CD34, and CD45 in BMSCs to be 94%, 96%, 91%, 2.7%, and 2.8%, respectively. Recombinant E1 region-deleted adenovirus type 5 carrying the human BMP-2 gene (Adv-hBMP-2) or β -galactosidase gene (Adv- β gal) with cytomegalovirus (CMV) promoter was constructed as previously described (Lou et al. 1999). Viral stocks were prepared in HEK-293 cells (ATCC, Manassas, VA) and the titer was determined by serial dilution and plaque-forming assay.

The BMSCs were infected by overnight incubation at 37°C in α -MEM containing Adv-hBMP-2 or Adv- β gal with a multiplicity of infection (MOI) of 200. After Adv-hBMP-2 gene transfer, the average dividing time of BMSCs was lengthened from 34.3 h to 41.1 h and 0.5–2% of cells were dead (measured by trypan blue staining). Alkaline phosphatase (ALP) staining showed that ALP-positive cells increased from 1–2% to 40–50% 10 days after gene transfer. After Adv- β gal gene transfer, the average dividing time was 35.8 h and 0.5% of cells were dead. X-gal staining showed 80–90% of BMSCs were X-gal positive and the duration of β gal gene expression was more than 60 days *in vitro*. Almost no cells died in non-transduced BMSCs.

Preparation of implants

On the eighth day after gene transfer in the gene-transduced groups, or the eleventh day after inoculation in the non-transduced group, BMSCs secreted much extracellular matrix and grew thickly, becoming membranous. Before implantation, they were torn directly from culture dishes using forceps. One of the membranous cellular masses was treated with trypsin and collagenase, to determine the quantity of cells. These gel-like cellular masses, carrying 3×10^8 autologous goat BMSCs, were incubated in FBS-free α -MEM at 37°C, and implanted to each bone defect as soon as possible.

Creation and repair of tibial bone defects in goats

Nineteen 1-year-old goats (11 males), which were grade 1 animals from Shanghai agricultural college, were licensed from the Shanghai science and technology committee. The range of animal weight was 18–30 kg. Animals were randomly divided into 4 groups with both sexes distributed as follows: I. Adv-hBMP-2 transduced BMSC group (7 goats, 4 male); II. Adv- β gal transduced BMSC group (5 goats, 3 males); III. non-transduced BMSC group (5 goats, 3 males); IV. untreated group (2 goats, 1 male). Ketamine and No. 846 anesthetic (Sumianxin) (Veterinary College, Changchun, China) were used for the anesthesia. The 2.1-cm segmental bone and periosteum of the right middle tibia in each goat was resected with a fretsaw. Groups I, II, and III were implanted with individual materials carrying autologous BMSCs mentioned in the grouping. Bone defects were stabilized with round external fixators consisting of 4 horizontal rings, 3 vertical screwed bars, and 6 horizontal K-wire pins (designed by the authors).

Radiography and histological examination

Radiographic examination was performed at 4, 8, 16, and 24 weeks after implantation. We evaluated the healing rates of bone defects on plain X-ray films at the end of week 24 post-surgery. Cortical continuity of both ends was used as the criterion of healing. 24 weeks after surgery, the animals were killed and specimens at the site of the primary tibial bone defects were harvested and examined. Each harvested specimen was divided equally into

two parts: the superior part was used to measure the biomechanical strength, while the inferior part was divided into 4 sagittal slices using a saw. The slices were decalcified, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H & E). The new bone area (NBA) of each slice was calculated by computerized histomorphometric analysis (FreeMax v.3.0, Zhongrui, Taipei, Taiwan) using a blind method. The new bone tissue in the initial bone defect was recognized by combination of automatic and manual methods. The average NBA of each specimen was calculated from the total NBA of 4 slices of the specimen divided by 4.

Biomechanical measurement

At week 24 after implantation, the superior parts of specimens in the primary tibial bone defects of each animal were sawn transversely to column-like structures, making sure the two sections were parallel (the shell of new cortical and trabecular bone was not removed during preparation). Average height and diameter were 1.23 ± 0.056 cm and 1.75 ± 0.11 cm, respectively. The samples were stored at -70°C and thawed before testing. The maximum vertical compressive loading (tested to failure) of the tibial segments was measured with an electronic omnipotent material mechanical tester (Z020, Zwick/Roell, Ulm, Germany). The metrical accuracy of loading and migration were 0.5% and 0.12 μm , respectively, and the loading speed was 5 mm/min. We used normal tibial segments of the opposite legs as normal control.

Assay of cellular immune response

The host cellular immune response against adenovirus was determined by T lymphocyte proliferation assay. In groups I, II, and III, 8 mL blood samples were collected from each goat before implantation and on days 14, 28, 49, and 120 after implantation. The lymphocytes were separated by centrifugation at 2,400 rpm with 70% Percoll solution in 15-mL centrifuge tubes and frozen in liquid nitrogen until use. Corresponding to each goat, wells of a 96-well plate (manufacturer) were used for infections according to the following three subgroups. Subgroup 1 signified Adv-hBMP-2 transduced BMSCs + lymphocytes; subgroup 2 signified Adv-hBMP-2 transduced BMSCs only; and subgroup

3 signified lymphocytes only. Thus, 8 wells were used for each subgroup. For subgroups 1 and 2, each well was inoculated with 5×10^4 autologous goat BMSCs in 200 μ L α -MEM, whereas 200 μ L α -MEM alone was added in the case of subgroup 3. 24 hours after inoculation, BMSCs were infected with Adv-hBMP-2 at a MOI of 200. 48 hours after infection, the BMSCs were treated with γ -radiation (^{60}Co) at a dosage of 100 Gy to stop mitosis and proliferation of the BMSCs. The lymphocyte suspension was adjusted to 10^7 cells/mL in RPMI 1640 medium. The α -MEM in each well was replaced with 200 μ L cellular suspension containing 2×10^6 lymphocytes (in the case of subgroups 1 and 3) or RPMI 1640 medium alone (for subgroup 2). After 5 days of incubation at 37°C , 1 μCi ^3H -TDR was added per well and incubated for 16 h. Cells were collected on Type 49 fiberglass filter papers to assay counts per minute (CPM) of ^3H -TDR in each well with a liquid scintigraphy instrument (Wallac 1450; Wallac Inc., Turku, Finland). We calculated the specific stimulation index (SI) of lymphocytes according to the following formula:

$$\text{SI} = \text{CPM (subgroup 1} - \text{subgroup 2)} / \text{CPM of subgroup 3 (Ding and Guan 1995)}.$$

Titer of plasma neutralizing antibody

The assay for plasma neutralizing antibody against adenovirus was performed according to a previously described method (Ganly et al. 2000). The goat plasma obtained from groups I, II, and III was incubated at 55°C for 30 min (waterbath) to inactivate complement. The plasma was diluted with α -MEM in five steps (plasma:total volume): 1:1, 1:5, 1:25, 1:125 and 1:625. The Adv-hBMP-2 solution was diluted to 100 plaque-forming units (PFU)/mL. 200 μ L viral dilution was mixed with 200 μ L diluted plasma (1000 μ L undiluted plasma for 5:1 (ratio of plasma vs viral dilution)) or α -MEM without plasma (as the negative control), and incubated at room temperature for 1 h. The mixture was added to HEK 293 cells at 70–80% confluence in 12-well plates (BD Falcon, Bedford, MA) and incubated at 37°C for 2 h. Then the mixture was replaced with a 0.5% agarose- α -MEM overlay. The number of plaques in each well was counted after 5 days of incubation at 37°C . The neutralizing antibody titer of each specimen was taken as the highest dilution to reduce the plaque number to 60% of the nega-

tive control. The data was transformed to logarithm with base 10^{-1} .

Statistics

We used Fisher's exact test to compare the healing rates among groups. Bartlett's test and normality test (W test) showed homogenous sample variances and normal distribution of the data concerning histomorphometry, maximum compressive loading, and titer of plasma neutralizing antibody, but the data on lymphocyte SI did not have homogenous sample variances. We used one-way ANOVA followed by least significant difference t (LSD-t test) test to compare the data on histomorphometry and maximum loading. Repeated-measures ANOVA (Least Squares Means) was used to compare the neutralizing antibody titer among groups, and among different times before and after implantation in the same group. We used non-parametric rank sum test (Kruskal-Wallis test) followed by Nemenyi test for the comparison of lymphocyte SI among the 3 groups, and M test (Friedman test) followed by comparison between each other was used for lymphocyte SI for different times before and after implantation in the same group. The 2-tail significance level (α) was 0.05.

Results

Radiographic examination

Radiographic findings after implantation of each group were as follows. (1) Adv-hBMP-2 group (group I): At weeks 4–8, bone defects were filled with continuous bony callus. At weeks 16–24, 6 of 7 bone defects had healed completely and the medullary cavity was partly recanalized (Figure 1A). (2) Adv- β gal group (group II) and non-transduced BMSC group (group III): At weeks 4–8, less bony callus formed in the defects. At weeks 16–24, the defect healing rates of groups II and III were 1/5 and 2/5, respectively (Figure 1B and 1C). (3) Untreated group: At weeks 4–8, little bone formation was found in bone defects. At weeks 16–24, there was nonunion in both bone defects. Fisher's exact test showed significant differences between the healing rates of group I and the controls (groups II, III, and IV) ($p = 0.005$).

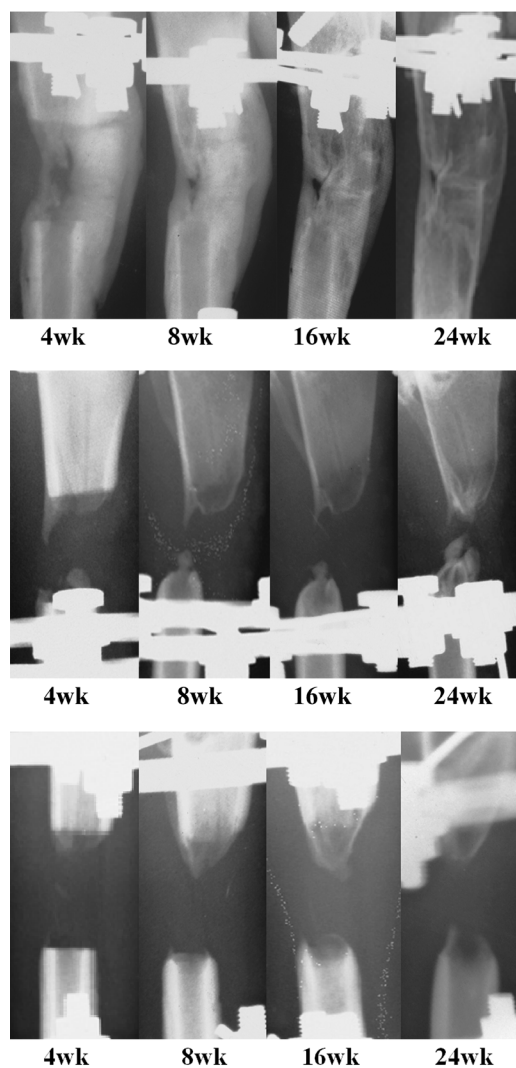


Figure 1. Radiographic examination of goat tibial bone defects after implantation of BMSCs. Adv-hBMP-2 group (top): weeks 4–8, much bony callus formed in the bone defect; weeks 16–24, bone defect healed completely and cortical bone formed with occurrence of medullary cavity. Adv-βgal group (middle) and non-transduced BMSC group (bottom): weeks 4–8, less bone formed in the bone defect; weeks 16–24, the bone defects of two groups had not healed, and the cut ends were blunt.

Histological examination

(1) Adv-hBMP-2 group: At week 24, much bony callus had formed in the bone defect. Part of new bone developed to cortical bone structure with medullary cavity formed. 6 of 7 bone defects showed complete healing (Figure 2A). (2) Adv-βgal group and non-transduced BMSC group: At week 24, the

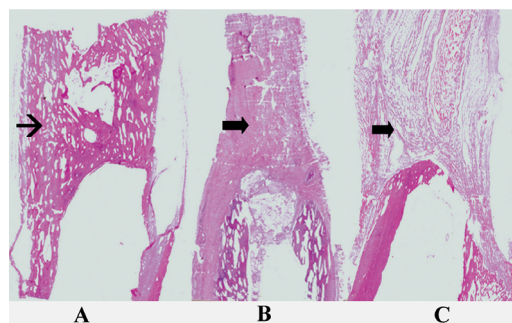


Figure 2. Histological examination of specimens from goat tibial bone defects at the twenty-fourth week after implantation of BMSCs. Adv-hBMP-2 group (A): Much bony callus (arrow) formed in the bone defect. Adv-βgal group (B) and non-transduced BMSC group (C): Bone defect was filled with fibrous tissue (arrows). (H & E staining, 2 ×).

Table 1. New bone area (NBA) in the goat tibial bone defects 24 weeks after treatment

Group	n	NBA (mm ²) ^a mean (SE)	LSD t-test p-value
I. Adv-hBMP2	7	148 (22)	I vs. II: 0.01
II. Adv-βgal	5	31 (11)	II vs. III: 0.5
III. BMSCs	5	48 (22)	I vs. III: 0.05
IV. Untreated	2	29 (6.2)	I vs. IV: 0.01

^a One-way ANOVA: $p = 0.002$.

defect healing rates of groups II and III were 1/5 and 2/5, respectively, and the rest of the defects were filled with fibrous tissue and a small amount of bony callus (Figure 2B and 2C). (3) Untreated group: Bone defects were filled with fibrous tissue and they did not heal. Histomorphometric analysis showed that the new bone area (NBA) in the Adv-hBMP-2 group was the highest among all groups. ANOVA and LSD t-test showed differences in NBA between group I and the controls (groups II, III, and IV) (Table 1).

Biomechanical measurement

The maximum compressive loading in group I was higher than in the control groups, and up to 64% of normal value. There were statistically significant differences in the comparison of maximum compressive loading between the Adv-hBMP-2 group and the controls (groups II, III, and IV), but we found no statistically significant differences in

Table 2. The maximum compressive loading of explants from goat tibial bone defects 24 weeks after treatment

Group	n	Maximum load (N) ^a mean (SE)	LSD t-test p-value
I. Adv-hBMP2	7	5354 (1097)	I vs. II: 0.003
II. Adv-βgal	5	1238 (396)	II vs. III: 0.6
III. BMSCs	5	1983 (952)	I vs. III: 0.02
IV. Untreated	2	776 (390)	I vs. IV: 0.02
Normal	19	8318 (521)	–

^a One-way ANOVA: $p = 0.01$.

Table 3. Specific stimulation index of lymphocytes in 3 groups before and after implantation. Mean (SE)

Group	I. Adv-hBMP-2 (n = 7)	II. Adv-βgal (n = 5)	III. BMSCs (n = 5)
Preop.	0.24 (0.29)	0.70 (0.26)	–1.4 (0.92)
Day 14	4.2 (1.3) ^{a,b,c}	–2.5 (1.7)	–0.31 (0.15)
Day 28	2.5 (0.96) ^d	2.3 (0.38) ^e	3.1 (0.64) ^f
Day 49	–0.061 (0.24)	1.8 (0.75)	–0.57 (0.40)
Day 120	–0.69 (0.71)	0.25 (0.14)	0.11 (0.20)

Kruskal-Wallis test and Nemenyi test:

Comparison to group II ^a

Comparison to group III ^b

Friedman test for comparison to preop:

^a $p = 0.01$

^b $p = 0.05$

^c $p = 0.03$

^d $p = 0.04$

^e $p = 0.04$

^f $p = 0.003$

biomechanical loading between groups II and III (Table 2).

Assay for cellular immune response

Non-parametric rank sum test showed that the lymphocyte SI in the Adv-hBMP-2 group on day 14 after implantation had increased significantly compared to those of groups II and III, and compared to its own level before implantation. On day 28 after implantation, the SI in three groups had increased relative to the corresponding SI before implantation of each group, but there was no statistically significant difference among groups. The results demonstrated that the Adv-hBMP-2 transduced BMSCs could stimulate the host cellular immune response against adenovirus, which lasted for 4 weeks (Table 3).

Table 4. Plasma titer of neutralizing antibody ($\log_{10}^{-1}$) in 3 groups before and after implantation. Mean (SE)

Groups	I. Adv-hBMP-2 (n = 7)	II. Advβgal (n = 5)	III. BMSCs (n = 5)
Preop.	0.24 (0.29)	0.70 (0.26)	–1.4 (0.92)
Preop.	0.96 (0.18)	0.84 (0.26)	0.14 (0.34)
Day 14	2.4 (0.23) ^{a,h}	1.4 (0.40) ^e	–0.18 (0.34)
Day 28	2.3 (0.20) ^{b,i}	2.1 (0.40) ^{f,l}	–0.42 (0.17)
Day 49	2.2 (0.22) ^{c,j}	1.9 (0.23) ^g	0.0 (0.17)
Day 120	2.3 (0.21) ^{d,k}	1.9 (0.23)	0.87 (0.52)

Repeated measures ANOVA:

Comparison to group III

^{a,b,c,f} $p < 0.001$,

^d $p = 0.008$

^e $p = 0.01$

^g $p < 0.001$

Comparison to preop:

^h $p = 0.005$

ⁱ $p = 0.002$

^j $p = 0.003$

^k $p = 0.01$

^l $p = 0.02$

Assay for neutralizing antibody in plasma

The low titer of neutralizing antibody against adenovirus was detected in the plasma of 11/17 goats before implantation; however, no statistically significant differences were found among the three groups. Repeated measures ANOVA showed that the titers in the Adv-hBMP-2 group on days 14, 28, 49, and 120 after implantation and in the Adv-βgal group 28 days after implantation had increased significantly compared to those before implantation. The titers in the Adv-hBMP-2 group (on days 14, 28, 49, and 120) and in the Adv-βgal group (on days 14, 28, and 49) after implantation were higher than those in group III (Table 4). The results showed that Adv-hBMP-2 transduced BMSCs could stimulate the host humoral immune response against adenovirus, which lasted for more than 17 weeks.

Discussion

BMSCs consist of a spectrum of different cells. There are two types of BMSC cells involved in osteogenesis: determined osteogenic precursor cells (DOPC) and inducible osteogenic precursor

cells (IOPC) (Fleming et al. 2000). Our results have shown that hBMP-2 gene transfer at a multiplicity of infection of 200 not only induced the differentiation of IOPCs to osteoblasts, but also inhibited the proliferation of BMSCs. The later effects were much stronger than those of Adv- β gal, so these changes were mainly the effect of hBMP-2, and adenovirus toxicity had less influence.

We used self-secreted extracellular matrix (ECM) as cellular carrier. In the membranous cellular mass, a great deal of ECM secreted by BMSCs themselves contains much collagen and non-collagenous proteins. Self-secreted collagen has good biocompatibility, superior biodegradability, no antigenicity, and can prevent loss of cells at the implant site. These characteristics facilitate the ingrowth and remodeling of new bone *in vivo*. The cooperation between BMP-2, collagen, and non-collagenous proteins plays an important role in the differentiation of BMSCs to osteoblasts and osteoinduction *in vivo* (Globus et al. 1995, Schneider et al. 2001, Suzawa et al. 2002, Xiao et al. 2002). In this study, we found that self-secreted ECM served as a good carrier for BMSCs in the repair of segmental tibial bone defects in goats, although self-secreted ECM has little osteoconductivity. This has not been reported previously in tissue engineering research. In another study, we demonstrated that osteoconductive scaffold could promote the osteogenesis, but the results of this research indicated that it was dispensable for the osteogenesis—provided the osteoinductivity of growth factors and osteogenic precursor cells was strong enough to initiate the osteogenesis (Xu et al. 2003).

Repair of bone defects in large animals, which imitates the clinical case, is an obstacle that must be overcome if BMP-2 gene transfer is to be translated from experimental studies to clinical trials. The larger the defect is, the more difficult it is to repair because of the difficulty in establishing the blood supply to cells in the center of bone substitute, especially in the tibial bone defect. In this study, the defect healing rate, the NBA, and the mechanical strength of the Adv-hBMP-2 group were superior to those of other groups. These results indicate that Adv-hBMP-2 gene transduced BMSCs possess strong osteoinductivity and can allow repair of segmental diaphyseal bone defects in large animals. Implanted BMSCs and surround-

ing endogenous MSCs could differentiate to osteoblasts and formed new bone under the influence of hBMP-2 from transgene expression. This osteogenesis was similar to the physiological process, and could be remodeled to cortical bone.

In this study, we used an *ex vivo* gene delivery method that could reduce the quantity of virus particles entering the body of the host. Also, the gene-transferred cells were implanted locally, not systemically, so the host immune response against viral proteins should be reduced. The results showed that adenovirus-mediated gene therapy could induce the temporary cellular and persistent humoral immune responses against adenovirus (for 4 weeks and for more than 120 days, respectively). To our knowledge, this is the first study to demonstrate the duration of cellular and humoral immune responses against adenovirus-mediated gene therapy. In our preliminary experiments, we found that a small number of lymphocytes had infiltrated the tissue of bone defects of the Adv-hBMP-2 group at 2 weeks after implantation, but had disappeared after 6–8 weeks (data not shown). The immune response is a disadvantage for hBMP-2 gene transfer since immune defense, especially the cellular immune response, can shorten the duration of hBMP-2 expression and damage the adenovirus-transduced BMSCs. It has been reported that adenovirus-mediated gene expression can last for 2–6 weeks in bone defects (Baltzer et al. 2000). We found that the duration of the cellular immune response was similar to the duration of gene expression in bone defects reported by Baltzer, so the duration of the cellular response may be an indication of *in vivo* duration of adenovirus-mediated gene expression. The duration of survival and termination of gene-transduced BMSCs *in vivo* requires further investigation. In another study, we compared the osteoinductivity in muscles of immunocompetent rabbits (using autologous cells) and athymic mice (with cellular immunodeficiency) by injection of Adv-hBMP-2 gene-transduced rabbit BMSCs (unpublished data). We found that the quantity of heterotopic bone formation in rabbits was less than in athymic mice, which indicated that the cellular immune response could reduce the effectiveness of gene transfer. The persistence of neutralizing antibody against adenovirus may reduce the effectiveness of repeated attempts at gene transfer. How-

ever, we succeeded in repairing goat tibial bone defects by Adv-hBMP-2 gene transfer even though the virus could induce an immune response. This also indicated that bone regeneration did not need long-term expression of the BMP-2 transgene. In this study, a low titer of neutralizing antibody against adenovirus was detected in the plasma of 12/19 goats before implantation. Whether the pre-existing antibody affects the effectiveness of gene transfer requires further study. There were some differences in SI between groups I and II. Further investigation is required to explain the reason for this. One possible reason is that at 2 weeks after implantation, the host cellular immune response in the Adv-hBMP-2 group was mainly induced by hBMP-2. The hBMP-2 concentration and lasting duration in local bone defects and serum, and whether it induces production of anti-hBMP-2 antibody, require further investigation. The reason for the increase in SI in the BMSC group may be that the cells have developed some mutations after long-term culture and/or that some fetal bovine serum may be carried into the host body.

Some researchers have reported that brief usage of immunosuppressors such as cyclophosphamide (Okubo et al. 2001) and FK506 (Tsuchida et al. 2003) can enhance the osteoinductivity of BMP-2 gene transfer. Other methods, including specific depletion of human anti-adenovirus antibodies (Rahman et al. 2001), anti-CD154 antibody (Stein et al. 2000), CTLA4-Ig, and new types of vector such as helper-dependent adenoviral vector (Abe et al. 2002, Li et al. 2003) or adeno-associated viral vector (AAV), might be used to enhance the osteoinductivity and decrease the damage to BMSCs. AAV apparently induces little immune response (Chen et al. 2003). Recently, vectors AAV-BMP-2 and 4 have been constructed and gene therapy experiments have shown promising results regarding induction of bone formation (Luk et al. 2003, Chen et al. 2003).

The prospects for application of BMP gene transfer are extensive. It can be used in the promotion of fracture healing, repair of bone defects and non-union, induction of anterior and posterior spinal fusion, prevention and treatment of loosening of arthroplasty, and enhancing attachment of tendon on the bone surface. This will provide a new set of possibilities for orthopedic practice.

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