Gene Marking in Adeno-Associated Virus Vector Infected Periosteum Derived Cells for Cartilage Repair

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ABSTRACT. ***Objective.*** To evaluate both the potential for transferring genes to periosteal cells using an adeno-associated virus (AAV) vector and the potential for gene expression after transplantation of those cells to a cartilage defect *in vivo*.

Methods. Periosteum was obtained from the tibia of 6-week-old rabbits and enzymatically digested. The isolated periosteum derived cells were cultured and the subconfluence cells were infected with a recombinant AAV expressing the LacZ gene (AAV-LacZ). Collagen gel containing the LacZ transferred, periosteum derived cells was transplanted into a full thickness articular cartilage defect in 10 rabbits.

Results. Infected cells still growing on the plate continued to express LacZ at least 12 weeks after AAV infection, with the highest percentage of LacZ positive cells reaching 74.4%. The LacZ positive cells were recognized at the transplant sites in 8 out of 10 knees.

Conclusion. Gene expression in periosteum derived cells was sustained *in vitro* for at least 12 weeks using the AAV vector, and for 2 weeks *ex vivo* after transplantation into a cartilage defect. (J Rheumatol 2002;29:2176–80)

Key Indexing Terms:
ADENO-ASSOCIATED VIRUS
PERIOSTEUM DERIVED CELLS
LACZ GENE
CARTILAGE

Mesenchymal stem cells are defined as multipotential cells capable of undergoing repeated mitotic division and differentiating into mesodermal tissue such as bone, cartilage, muscle, adipose tissue, and tendon under appropriate conditions[1]. Periosteal cells are thought to be a type of multipotential cell found in postnatal organs. Their chondrogenic property has made periosteal cells attractive for cartilage repair and periosteal grafts, leading to their use both experimentally and clinically for the treatment of cartilage defects[2].

To promote cartilage repair, implanted cells may need stimulation from various growth factors, especially transforming growth factor-ßI (TGF-ßI)[3]. However, it may be difficult to achieve or maintain a bioactive level of growth factors in a cartilaginous lesion with a single exposure *in vivo*. Sustained gene expression of growth factors *in vivo* is needed to maintain their stimulatory levels and achieve satisfactory cartilage repair.

Recently, there have been several attempts to treat arthritis with gene therapies using vectors such as adenovirus[4], retrovirus[5], herpes simplex virus, or nonviral methods[6] to perform the gene transfer. Among them, adeno-associated virus (AAV) is recognized as having several advantages over the others: an authentic capability of infecting many kinds of cells, no virulency, integration of the transduced gene into the host chromosome, and longevity of gene expression[7]. However, there have been few reports discussing gene delivery to synovial cells or chondrocytes using an AAV vector for the treatment of joint disorders[8-12]. We investigated the ability of the AAV vector to transfer a gene to periosteum derived cells *in vitro* and also the possibility for the gene transferred cells to repair a cartilage defect.

MATERIALS AND METHODS

Periosteum was obtained from the medial side of the tibia of 6-week-old Japanese white rabbits, and digested with 0.05% trypsin-EDTA and 0.25% collagenase. Subsequently, about 3×10^6 cells were obtained from one piece of the periosteum. The liberated cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) and supplemented with 10% fetal bovine serum, penicillin, and streptomycin.

The AAV vector expressing LacZ gene (AAV-LacZ) was produced as described[13]. Briefly, human fetal kidney cells (293 cells) cultured in DMEM:nutrient mixture F-12 (1:1) (DMEM/F-12; GibcoBRL, New York, NY, USA) that was supplemented with 10% fetal bovine serum (FBS) were used for conjugation of the AAV vector and plasmid LacZ. The 293 subconfluent cells were cotransfected by the calcium phosphate coprecipitation

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method with pLacZ, pIM45, and pladeno-1 to produce the AAV inducing LacZ gene (AAV-LacZ). After 48 h, the cells were harvested and lysed in Tris buffer (10 mM Tris-HCl, 150 mM NaCl, pH 8.0) through 3 cycles of freezing and thawing. One round of sucrose precipitation and 2 rounds of CsCl density gradient ultracentrifugation were performed to isolate the AAV-LacZ from the lysates. The vector titer was determined by quantitative DNA dot-blot hybridization of the Dnase-I resistant fraction.

As a preliminary experiment, the percentage of LacZ positive cells attendant with each virus titer was investigated. The subconfluent cells in a 96 well dish were infected with AAV-LacZ in titers of 10^3, 10^4, 10^5, 10^6 particles/cell, and incubated 1 h. One week after infection, the percentage of LacZ positive cells was calculated and averaged for each virus titer (Figure 1). The mean percentage of LacZ positive cells at each titer was 49.0% ± 9.2% at 10^3, 72.1 ± 4.1% at 10^4, 80.4 ± 8.2% at 10^5, 83.1 ± 5.4% at 10^6 particles/cell, with the optimum titer determined to be 10^5 particles/cell.

After the subconfluent cells in subculture dishes were washed with phosphate buffered saline (PBS), AAV-LacZ (10^5 particles/cell) was added, incubated 1 h, and then cultured for 1 week. One week after infection some of the harvested cells were mixed with 80 μl of 0.25% collagen gel (DME-02; Koken Co. Ltd., Tokyo, Japan) and the cell density was adjusted to 4×10^6/ml. The cells embedded in the collagen gel were cultured for 2 days in preparation for transplanting to a cartilage defect.

Intravenous pentobarbital (0.6 ml/kg) was used to anesthetize ten 8-week-old Japanese white rabbits for surgery. One knee of each rabbit had a full thickness defect 5 mm diameter by 3 mm deep drilled into the femoral patellar groove, which was then filled with the collagen gel containing the periosteum derived cells. Periosteum harvested from the contralateral tibia was used as a patch and fixed at the edge of the defect. The other knee received a sham operation as a control, and the defect was filled with either cell-free collagen gel or gel containing non-AAV infected cells. The rabbits were allowed to move freely immediately after surgery.

Expression of LacZ in cells remaining in the original culture was evaluated at 3 days and 1, 2, 4, and 12 weeks after AAV-LacZ infection. Cells transplanted into the cartilage defect were evaluated for LacZ expression at 1 and 2 weeks after transplantation. After fixation with PBS containing 2% formaldehyde and 0.2% glutaraldehyde, the specimens were directly stained with X-gal solution. Under a microscope at 100× magnification, 4 visual fields were randomly selected. The total number of cells and the number of LacZ positive cells were counted to determine the percentage of LacZ positive cells.

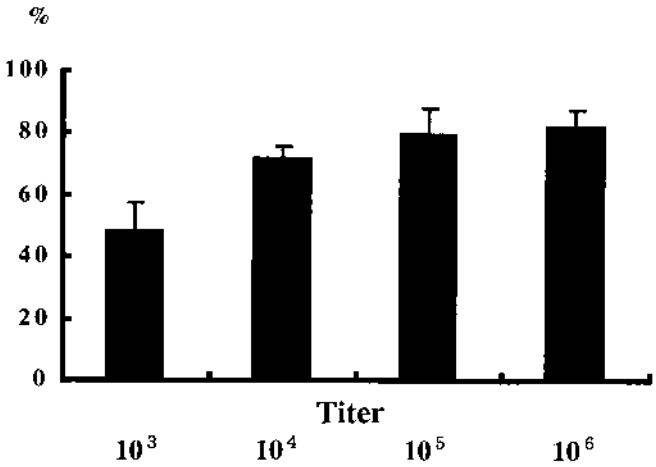

Figure 1. Percentage of LacZ positive cells at different AAV titers: 49.0% ± 9.2% at 10^3, 72.1 ± 4.1% at 10^4, 80.4 ± 8.2% at 10^5, 83.1 ± 5.4% at 10^6 particles/cell.

RESULTS

LacZ expression in vitro. Many blue stained LacZ positive cells were observed in the culture dishes, and this number increased gradually with time (Figure 2). The mean percentage of LacZ positive cells per total cells was 54.2 ± 10.2% at 3 days after infection with AAV-LacZ (Figure 3A), 68.2 ± 3.8% at 1 week (Figure 3B), 73.9 ± 6.0% at 2 weeks, and 74.4 ± 6.9% at 4 weeks (Figure 3C, 3D). By 12 weeks postinfection, the number of LacZ positive cells decreased to 53.2 ± 11.7% (Figure 3E), but gene expression was still strong. There was no cytotoxicity or other side effects of the virus, and there was no difference in cell growth or morphology between AAV infected cells and noninfected cells *in vitro* (data not shown).

LacZ expression after transplant in vivo. The cells implanted in the cartilage defect were enucleated, snap frozen in OCT compound, sectioned thinly with a cryotome, and stained with X-gal. Eight out of 10 rabbits had transplanted cells that strongly expressed LacZ (Table 1). One week after the transplant, LacZ positive cells were observed beneath the periosteum patch (Figure 4A), and the expression of LacZ was sustained for at least 2 weeks after transplant (Figure 4B).

DISCUSSION

One advantage of the AAV vector is its ability for sustained gene expression because of its integration of the target gene

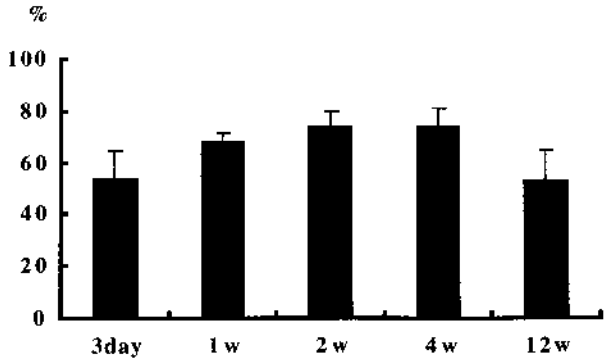

Figure 2. Percentage of LacZ positive cells at different times after infection. The percentage increased gradually with time reaching a maximum at 4 weeks (w: week).

Table 1. LacZ was expressed at 80% of all transplantation sites (n = 10).

Time After Transplantation	Knees with Transplants	Knees with LacZ + Cells (%)
1 week	5	4 (80)
2 weeks	5	4 (80)
Total	10	8 (80)

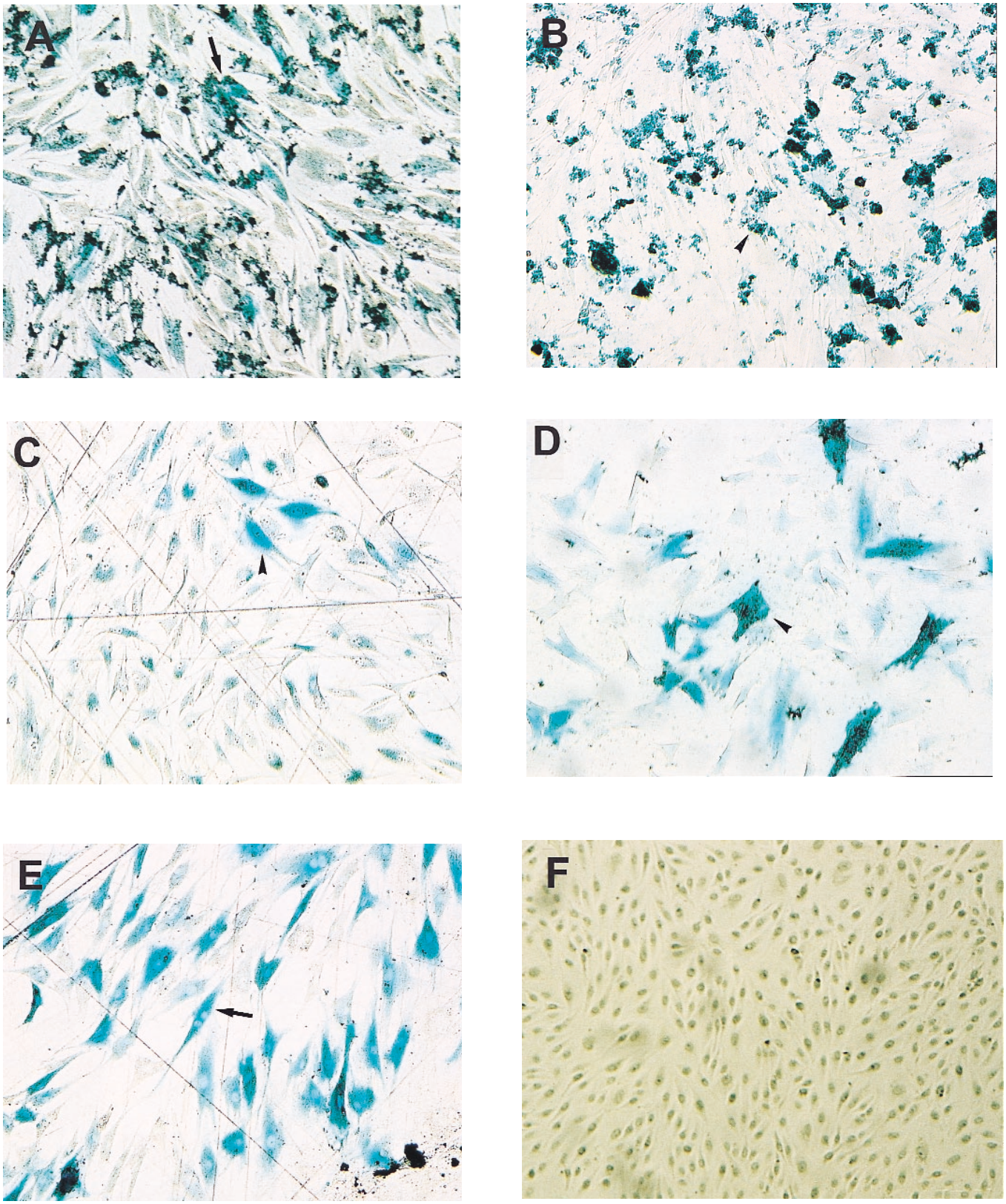

Figure 3. Photomicrographs of periosteum derived cells on culture plate stained with X-gal (100× original magnification). The X-gal staining was performed at 3 days (A) and at 1 (B), 2 (C), 4 (D), and 12 (E) weeks after AAV-LacZ infection. LacZ expression was sustained strongly for at least 12 weeks *in vitro*. Arrows indicate LacZ positive cells. LacZ positive cells were not seen in the control cells (F).

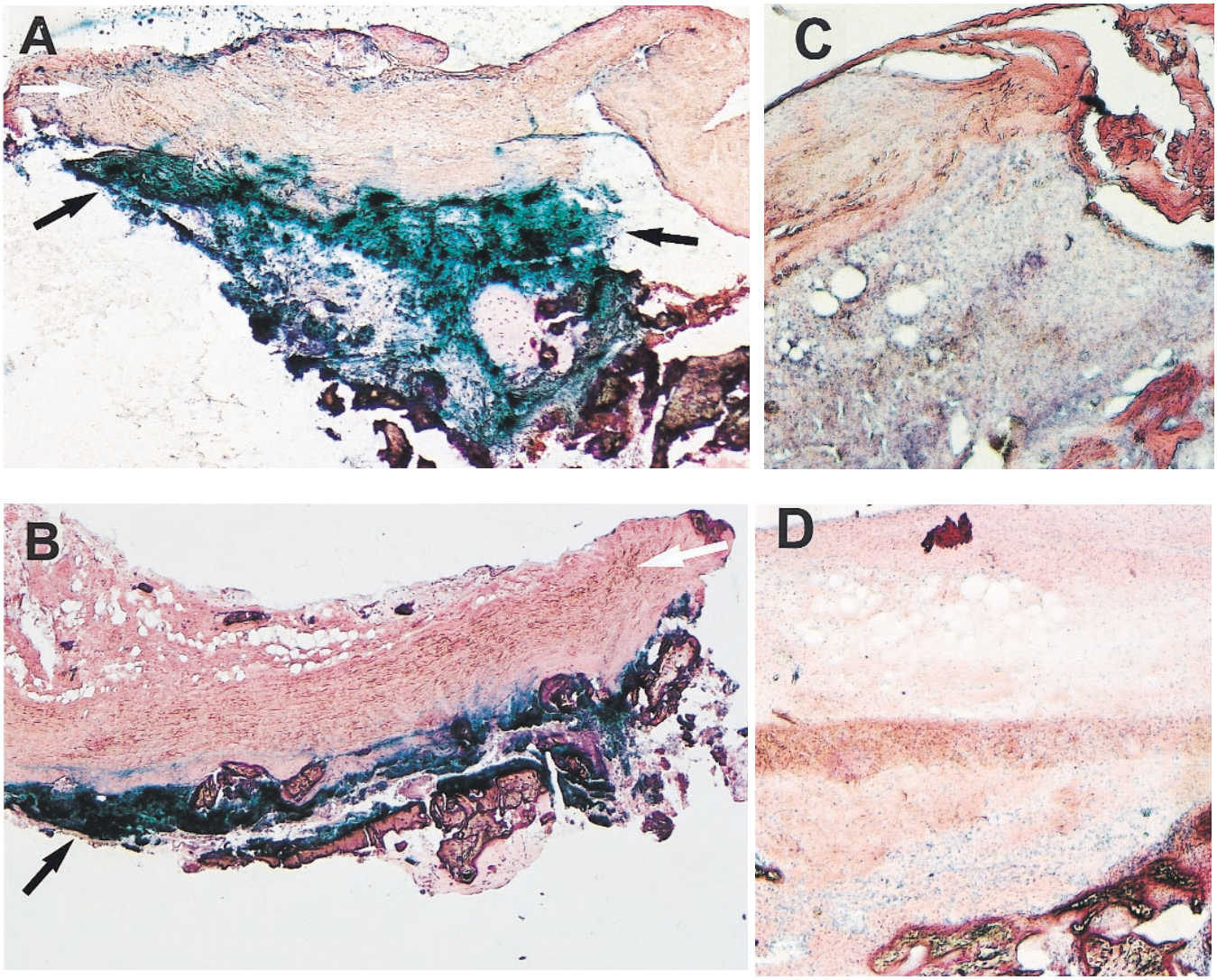

Figure 4. Microphotograph of a specimen obtained from a knee with transplanted periosteum derived cells embedded in collagen gel (20× original magnification). X-gal and H&E stainings were performed on each specimen using frozen sections. The transplanted cells grew at the transplant site and expressed LacZ under the periosteum patch when examined 1 and 2 weeks after transplant (A, B). White arrows indicate the periosteum patch, black arrows indicate LacZ positive periosteum derived cells. In control knees treated with cell-free collagen gel (C) or collagen gel with noninfected cells (D) there were no LacZ positive cells.

into the host chromosome[14]. We confirmed continued gene expression for 12 weeks *in vitro*, whereas in a previous study using another vector, the longest term of transferred gene expression was about 8 weeks[4,5], which indicates the superior ability of the AAV vector for sustained gene expression.

The advantage of *ex vivo* over *in vivo* gene delivery is the ability to limit the area of gene expression to the cartilage defect alone. Because of the strong infection potential of AAV, it is not certain where the AAV will infect using *in vivo* gene transfer. For example, synovium is easier to infect than cartilage since AAV has difficulty passing through the extracellular matrix[8-10]. Kang, *et al* performed *ex vivo* gene transfer to chondrocytes using a retroviral vector and confirmed LacZ expression for 4 weeks *in vivo*, but the percentage of LacZ positive cells was less than 10%[15], whereas this study had considerably more LacZ positive cells confirmed at the transplant site.

In a previous study using an AAV vector, the synovium[8,9,11,12] and the cartilage[9,10] were chosen as the target tissue for gene transfer. However, there have been no studies on gene transfer to periosteum derived cells using an AAV vector both *in vitro* and *in vivo*. We used periosteum derived cells, which can be obtained easily and maintain their chondrogenic potential throughout expansion regardless of donor age[16]. Periosteum derived cells were stained strongly at immunohistochemistry using anti-fibroblast antibody (Biomeda Corp., Hayward, CA, USA), so immunohis-

tochemical type of these cells was defined to be fibroblasts (data not shown).

We confirmed sustained gene expression in periosteum derived cells *in vitro* using an AAV vector, and also confirmed the usefulness of AAV as an *ex vivo* gene transfer agent, making this the first study demonstrating gene transfer to periosteum derived cells using an AAV vector. In the future, this method may be used for repairing cartilage defects or for treating osteoarthritis.

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