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ORIGINAL ARTICLE

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Efficient gene delivery to articular cartilage using electroporation

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Abstract Effective in vivo gene transfer into articular cartilage has not yet been established. Since chondrocytes are embedded within a rich extracellular matrix, various gene transfer methods have failed to introduce genes into deeper layers of the articular cartilage. In this study, we developed new superfine pointed needle electrodes for in situ electroporation (EP), and investigated the efficiency of gene transfer into articular cartilage with different degrees of degeneration. Full-thickness articular cartilage slices were obtained from the knee joint of a 3–4-month-old rabbit. The cartilage tissues were treated briefly with trypsin to partly remove matrix proteoglycan. Human articular cartilage with different grades of degeneration was also used. For EP, the articular cartilage surface was soaked in a solution containing green fluorescent protein (GFP) plasmid. Then, the superfine pointed 7-needle electrodes were gently stabbed into the surface layer of the articular cartilage and the gene was transfected by an electroporator. GFP expression was examined by immunohistochemical analysis. Cartilage tissue was successfully transfected with the GFP gene by the electrodes and EP. Transfection efficiency was enhanced by depleting the matrix proteoglycan in rabbit articular cartilage. Chondrocytes in the deeper layer of the articular cartilage were also transfected and expressed GFP. In human osteoarthritic cartilage, ca. 30% of the cells in the deeper layer were transfected by selecting optimal EP conditions. No adverse effects of EP on damaged articular cartilage were obvious from histological analysis or TUNEL staining. The results indicated that EP-mediated in vivo gene transfer into articular cartilage may provide a useful therapeutic strategy to treat cartilage degeneration.

Key words Articular cartilage · Electroporation (EP) · Gene transfer

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Introduction

It is well known that once the articular cartilage is damaged, the capacity for self-repair is limited. This is partly due to the absence of blood vessels in articular cartilage and the inability of the remaining chondrocytes to divide and maintain sufficient synthetic activity to reconstitute the matrix architecture. Various methods have been employed to inhibit cartilage breakdown and to enhance the reparatory process, e.g., the inhibition of proteolytic activity, anti-inflammatory cytokine therapy, the use of growth factors for chondrocyte proliferation and matrix synthesis, the inhibition of chondrocyte cell death, cell-based therapies for cartilage regeneration, and gene therapy.[1–7]

Recent progress in gene transfer techniques has made gene therapy one of the therapeutic strategies used to treat joint disorders. Current in vivo gene transfer methods include cationic liposomes, viral vectors, HVJ-liposome, injection of plasmid DNA, and electroporation (EP). Each of these methods has various advantages and/or disadvantages in terms of gene transfer efficiency, toxicity, inflammatory response, need for cell replication, and technical simplicity. For clinical applications in the treatment of damaged articular cartilage and related disorders, it is desirable to transfer exogenous genes into articular cartilage by a direct in vivo approach. However, the direct in vivo approach raises difficult problems. Chondrocytes do not usually undergo replication, and are embedded in the rich extracellular matrix. This feature of articular cartilage seems to have hampered in vivo gene transfer into chondrocytes.[7,8] Although cells in the surface layer of cartilage were transfected by adenovirus, adeno-associated virus, and HVJ-liposome vectors, cells in the deeper layer were resistant to gene transfer.

EP is a well-established method for introducing exogenous molecules or genes into cells by high-voltage electric pulses. Several studies of gene transfer by EP with plasmid injection demonstrated the high efficiency of in vivo and in situ gene transfer into liver, corneal endothelium, skin, skeletal muscle, and tumor tissues.[9–14] EP also achieved the long-term expression of a functional transgene in a brain with limited damage to the tissue.[15] We therefore examined whether or not EP can be applied to gene transfer into articular cartilage in vivo. We developed new electrodes with an array of superfine pointed needles for partial penetration into the articular cartilage surface. Using an in vivo-simulating condition, we transferred the green fluorescent protein (GFP) gene by EP into rabbit and human articular cartilage in situ. After EP, chondrocytes in the deep layer, as well as in the superficial-to-middle layers, of the cartilage expressed GFP, suggesting that EP with the newly developed electrodes is a useful procedure for the in vivo delivery of genes into articular cartilage.

Materials and methods

Plasmid

The GFP gene (pEGFP-C1, Clontech, Palo Alto, CA, USA) was used for the gene transfer. The plasmid was amplified, purified using the Qiagen Plasmid Maxi Kit (Qiagen, Valencia, CA, USA), and adjusted to a concentration of 1 mg/ml in phosphate-buffered solution (PBS)(−) for this study.

Articular cartilage tissues

Experiment 1

Rabbit articular cartilage was obtained from the knee joint of male adolescent (3–4 months old) Japanese white rabbits, weighing approximately 3 kg. After exposure of the knee joint, full-thickness articular cartilage with some thin subchondral bone was dissected from the femoral and tibial condyles, and washed with PBS. To partially remove matrix proteoglycan, the cartilage specimen was soaked in 0.125% trypsin solution in PBS at room temperature. The specimens were classified into three groups (n = 3 in each group): no-treatment group, 20-min trypsin treatment group, and 120-min trypsin treatment group.

After treatment with trypsin, the specimens were washed with PBS and placed in each well of a 6-well culture plate containing 500 μl GFP plasmid solution (1 mg/ml PBS). To simulate in vivo gene transfer, the articular cartilage was placed upside-down in order to soak the cartilage surface only with the DNA solution and incubated for 15 min at room temperature before EP (Fig. 1).

Experiment 2

Human cartilage tissues were obtained from patients with osteoarthritis at the time of knee arthroplasties. Cartilage tissue with various degrees of degeneration was obtained and classified into five grades by macroscopic appearance as follows[16]: grade 0, almost normal (n = 3); grade I, superficial fibrillation or surface flaking (n = 3); grade II, fissuring or fibrillation of less than half the thickness of the articular cartilage (n = 3); grade III, fasciculation, fragmentation, or degeneration greater than half the thickness of the articular cartilage (n = 3); grade IV, erosion to the subchondral bone. The tissue that belonged to grade IV was not included in this study. The cartilage tissues were washed with PBS, placed in each well of a 6-well culture plate containing 500 μl GFP plasmid solution (1 mg/ml PBS) and the surface was soaked for 15 min at room temperature before EP. As a control, cartilage tissue was soaked with PBS without GFP plasmid for 15 min in the same manner.

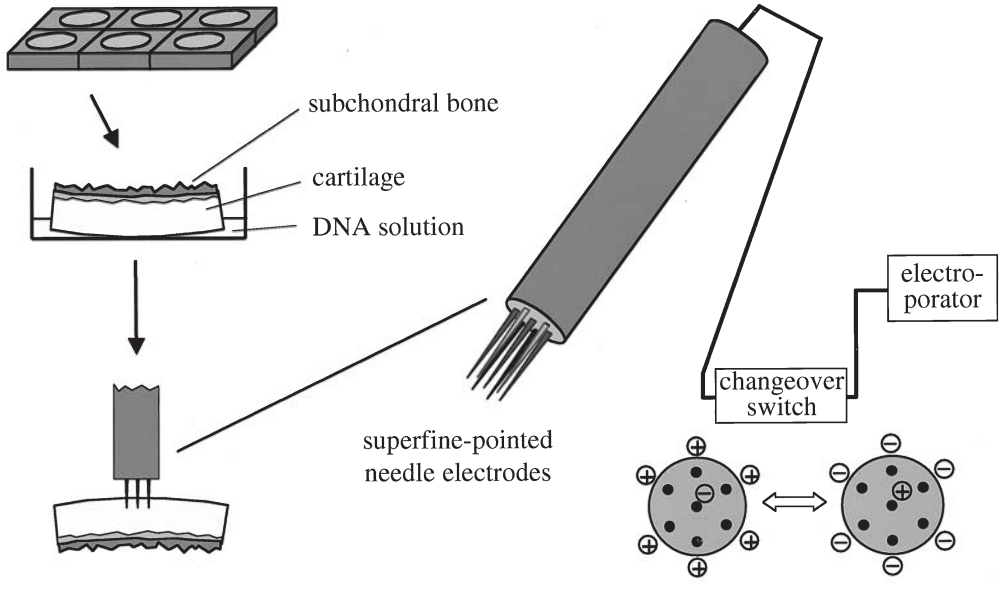

Fig. 1. Superfine pointed 7-needle electrodes and electroporation into articular cartilage. The articular surface was placed upside-down to soak the surface in the DNA solution for 15 min, followed by stabbing of the electrodes and electroporation

Gene transfer into articular cartilage by EP

For in vivo simulation of gene transfer into cartilage, we have developed a new type of electrode with an array of superfine pointed needles for stabbing (or penetration) into the articular cartilage surface (see Fig. 1). After soaking the cartilage surface with PBS containing plasmid DNA, the rabbit and human cartilage tissues were pulsed immediately with the electrodes using electroporator CUY 21 (BTX, San Diego, CA, USA). EP was set up in two different conditions after a preliminary experimental analysis of transfection efficiency, i.e., 8 square-wave pulses, pulse length 50 ms, 2 Hz, and a voltage of 20 or 50 V. EP at a single site was carried out twice by changing the electric field with a switching device. The whole area of the articular surface was treated by stabbing the electrodes and EP. After gene transfer, tissues were transferred into a DMEM medium containing 10% fetal calf serum (FCS) and antibiotics (100 units/ml penicillin G and 100 mg/ml streptomycin) and cultured for 2 days. The specimens were rinsed with PBS, fixed in 4% paraformaldehyde for 2 h at room temperature, dehydrated in 100% ethanol, and then embedded in paraffin. GFP expression was analyzed by immunohistochemical staining using GFP monoclonal antibody (Clontech).

Histological analysis

Serial sections with a thickness of 5 μm were stained with safranin-O/fast green. To examine the expression of GFP, immunohistochemical staining for GFP was performed using standard procedures. Briefly, the sections were deparaffined, treated with 0.3% H_2O_2 in methanol to block endogenous peroxidase for 30 min at room temperature, and then rinsed with PBS. A GFP monoclonal antibody was used as a primary antibody, and incubated overnight at 4°C. PBS was substituted for primary antibody as a negative control. Immunoreactivity was detected using biotinylated horse antimouse IgG (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature, followed by an avidin–biotin kit (Vector Laboratories) for 30 min at room temperature. The signal was visualized as a brown reaction product of diaminobenzidine (DAB) and H_2O_2. Methyl green staining was used for counter-staining.

TUNEL assay

To examine the degree of tissue damage caused by EP, chondrocytes were labeled using a TUNEL (terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-digoxigenin nicked-end labeling) peroxidase apoptosis detection kit (Apoptag Peroxidase Kits, Intergen, Gaithersburg, MD, USA) according to the manufacture's protocol. Briefly, 5-μm-thick deparaffinized sections were pretreated with proteinase K (20 μg/ml) for 15 min at room temperature. After washing with PBS, the endogenous peroxidase was inactivated in 3% H_2O_2 for 5 min, followed by incubation with TdT enzyme at 37°C for 1 h. Sections were then incubated with antidigoxigenin conjugate at room temperature for 30 min, followed by incubation with DAB solution. Methyl green staining was used for counter-staining.

Estimation of transfection efficiency

To estimate the efficiency of transfection by EP, we calculated the number of GFP-positive cells in ten randomly selected fields under light microscopy. The percentage of GFP-positive cells was estimated as GFP-positive chondrocyte number/all chondrocytes in each section, and determined as the average of each percentage of ten fields.

Statistical analysis

Welch's *t*-test was performed to analyze the results using Microsoft Excel 2000. Values are expressed as mean and SEM.

Results

Expression of GFP in rabbit articular cartilage

To examine gene transfer and expression in rabbit articular cartilage by EP, tissue specimens were observed by light microscopy and immunohistochemical staining specific for GFP. In normal articular cartilage, GFP-positive cells were hardly detected after EP at 20 V. At 50 V, however, a low number of GFP-expressing cells was detected in the deep layer, as well as in the superficial-to-middle layer of articular cartilage (Fig. 2D). Since changes in cartilage matrix composition may affect transfection efficiency, the tissue was treated with trypsin for 0, 20, and 120 min before EP. In trypsin-treated articular cartilage that lost proteoglycan and safranin-O staining, an increased number of GFP-expressing chondrocytes was obvious (Fig. 2F). Although the number of GFP-positive cells was not very high, it was noteworthy that chondrocytes in the deeper layer were also transfected.

The number of GFP-positive cells was calculated in the sections, and Fig. 3 shows the transfection efficiency into the superficial-to-middle layer and the deep layer of cartilage after EP. At 20 V, about 12% of the chondrocytes in the superficial-to-middle layer was transfected when treated with trypsin, although the GFP expression in the deep layer was low. At 50 V, however, there was improved transfection efficiency in normal cartilage. In addition, cells in the deep layer of the cartilage were also transfected and expressed GFP, irrespective of trypsin treatment.

Gene transfer into human cartilage with various degrees of degeneration

The GFP gene was not efficiently transfected into grade 0 human articular cartilage that has a normal surface appearance (not shown). However, relatively good transfection was observed in grades I–III cartilage, suggesting that

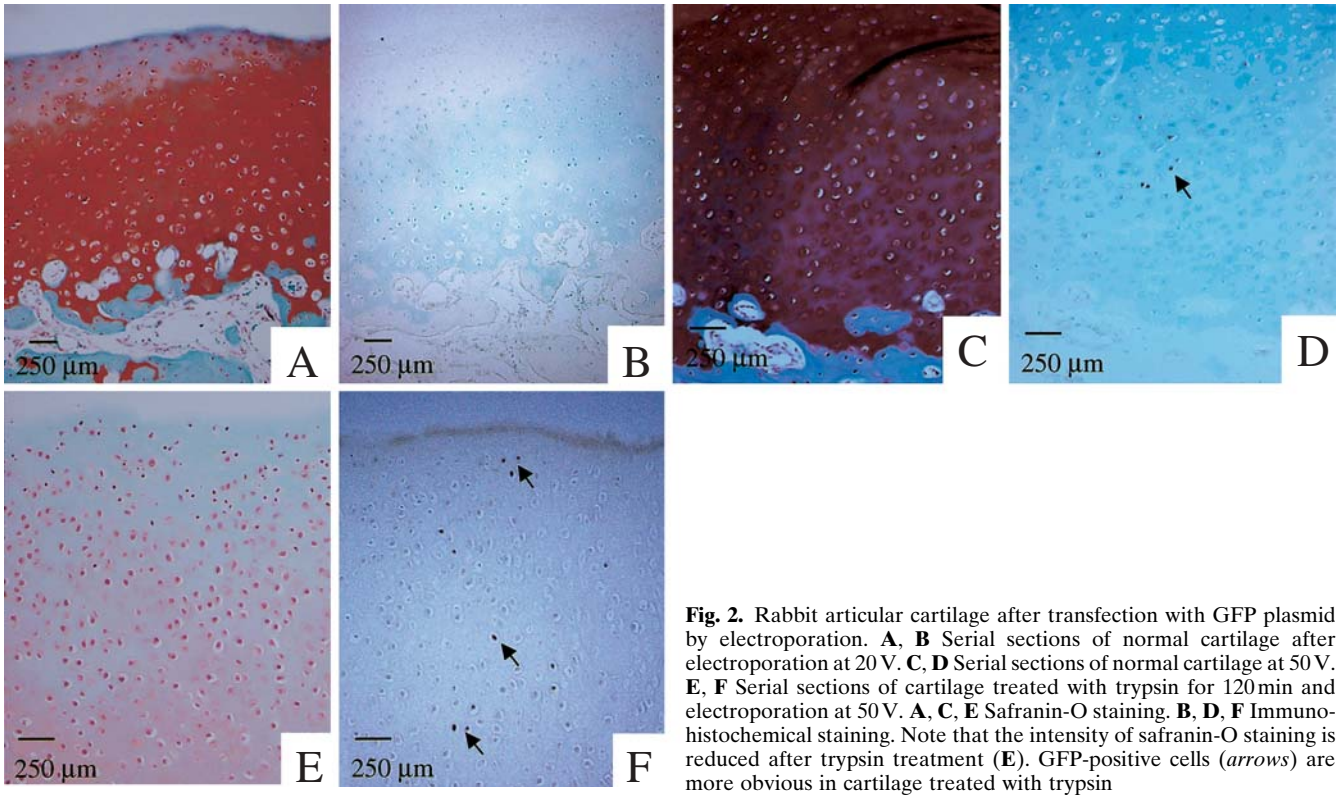

Fig. 2. Rabbit articular cartilage after transfection with GFP plasmid by electroporation. **A**, **B** Serial sections of normal cartilage after electroporation at 20 V. **C**, **D** Serial sections of normal cartilage at 50 V. **E**, **F** Serial sections of cartilage treated with trypsin for 120 min and electroporation at 50 V. **A**, **C**, **E** Safranin-O staining. **B**, **D**, **F** Immunohistochemical staining. Note that the intensity of safranin-O staining is reduced after trypsin treatment (**E**). GFP-positive cells (*arrows*) are more obvious in cartilage treated with trypsin

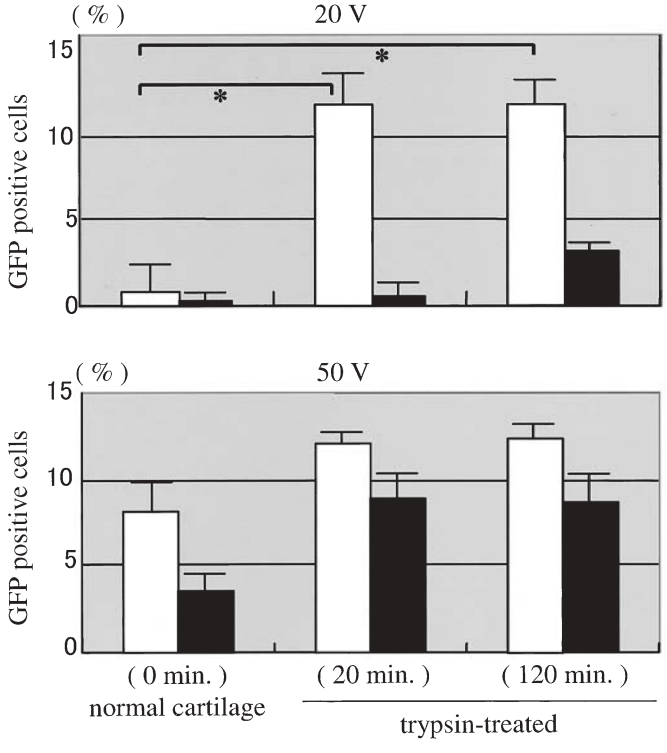

Fig. 3. Efficiency of GFP-expression in rabbit articular cartilage. A cartilage specimen was treated with trypsin to remove proteoglycan for 0, 20, or 120 min before electroporation. The results represent the average percentage of GFP-positive cells after gene transfer. Error bars show ±SEM. The *upper panel* shows the results of electroporation at 20 V, and the *lower panel* at 50 V. *White bars* indicate GFP expression in the superficial-to-middle layers within cartilage tissue, and *black bars* indicate expression in the deep layer. *Asterisk*, $P < 0.01$

fibrillated cartilage that has lost proteoglycan is a good recipient of gene transfer by EP (Fig. 4). Chondrocytes in the deeper layer of damaged cartilage and in the cell clusters were also transfected. Transfection efficiency was then estimated by counting the GFP-positive cells in randomly selected sections. At 50 V, as many as 40% of the cells in the middle layer were transfected and expressed the GFP gene (Fig. 5). Cells in the deep layer of the degenerated cartilage were also transfected and expressed GFP.

Cartilage matrix damage by EP

Although surface stabbing with the superfine pointed needle electrodes did not cause any apparent morphological damage in trypsin-treated rabbit cartilage or grades I–III human cartilage, it caused mild indentations in "normal" cartilage. No loss of safranin O staining was caused by EP, and the cells around the stab site expressed GFP (Fig. 6). However, nonspecific staining was observed around the penetration site. This may be due to the subtle degeneration of collagen fibril within the matrix. Therefore, EP may not be warranted for normal cartilage.

Damage to chondrocytes by EP

To detect cell death or DNA damage after gene transfer by EP, TUNEL staining was performed in every rabbit and human cartilage specimen. Figure 7 shows a representative photograph. Osteoarthritic cartilage usually shows the presence of some percent of apoptotic chondrocytes. However, in all human cartilage specimens, there was no increase in

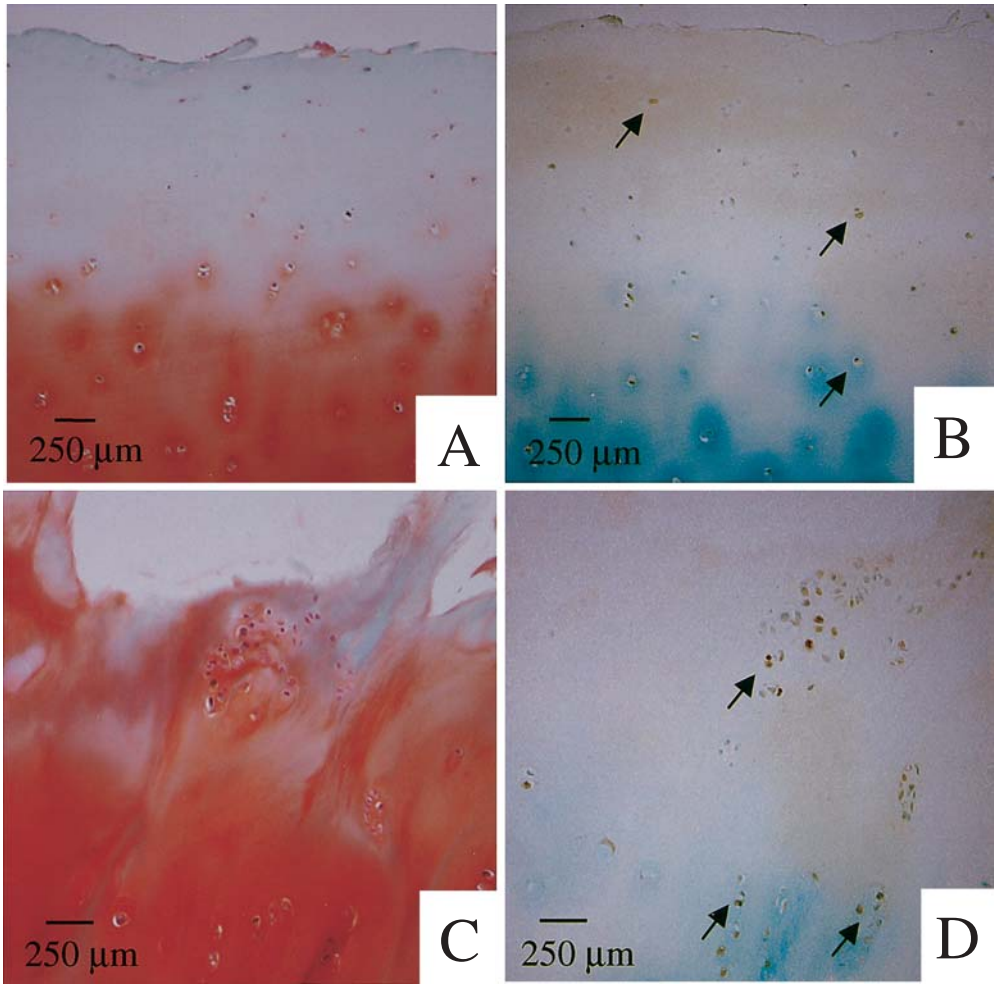

Fig. 4. Representative photograph of human articular cartilage after GFP gene transfer by electroporation. Grade III cartilage (**C**, **D**) shows higher transfection efficiency than grade I cartilage (**A**, **B**). **A**, **C** Safranin-O staining. **B**, **D** Immunohistochemical staining for GFP

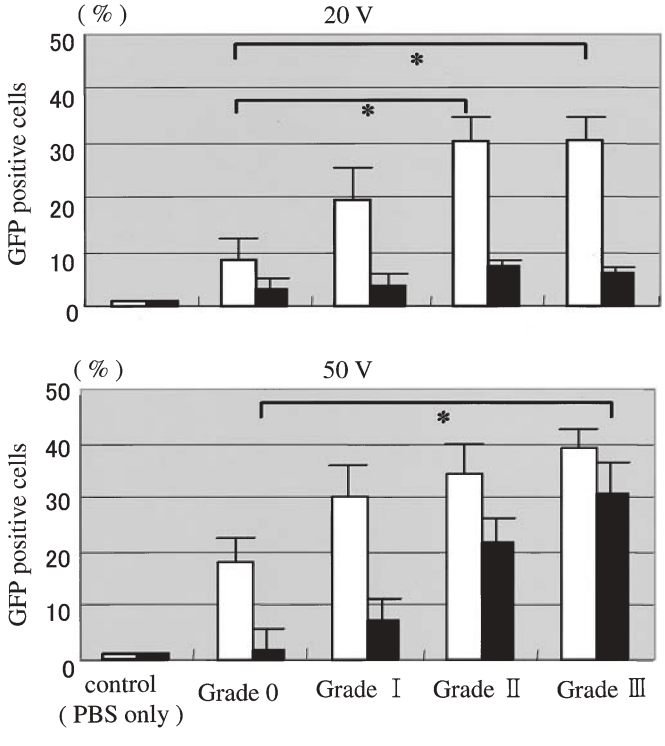

Fig. 5. Efficiency of GFP expression in human articular cartilage. The results represent the average percentage of GFP-positive cells after gene transfer by electroporation. Error bars show ±SEM. The *upper panel* shows the results of electroporation at 20 V, and the *lower panel* at 50 V. *White bars* indicate GFP expression in the superficial and/or middle layer within cartilage tissue, and *black bars* indicate expression in the deep layer. Note that the superficial layer and part of the middle layer were often eroded or fragmented in the grade III cartilage. *Asterisk*, $P < 0.01$

the number of TUNEL-positive cells at the site where GFP-positive cells were present, suggesting that EP did not cause any apparent cell damage to chondrocytes.

Discussion

Our present study demonstrated several advantages of EP-mediated gene transfer for rabbit articular cartilage and human osteoarthritic cartilage. In a rabbit model, we treated the cartilage with trypsin to remove proteoglycan,[17,18] and showed that this treatment enhanced gene transfer into cartilage. This suggested that degenerated articular cartilage is more susceptible to gene transfer. In fact, EP to human osteoarthritic cartilage showed an increase in

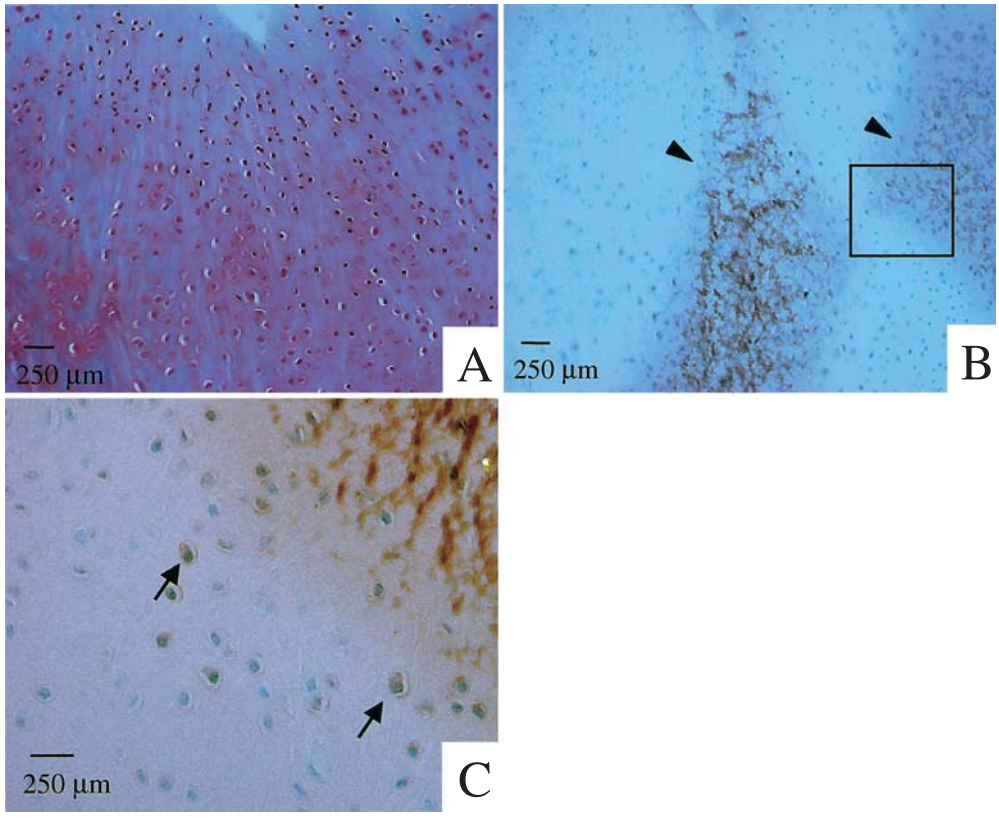

Fig. 6. Cartilage matrix damage by electroporation. Stabbing of the superfine pointed electrodes caused mild indentations on the surface of normal rabbit articular cartilage. The intensity of safranin-O staining at the needle penetration site was not different from that in the surrounding area (**A**). **B** Immunohistochemical staining. **C** Higher magnification of the rectangular area in (**B**). There was some nonspecific staining (*arrow heads*), in addition to GFP-expressing cells (*arrows*) (**C**)

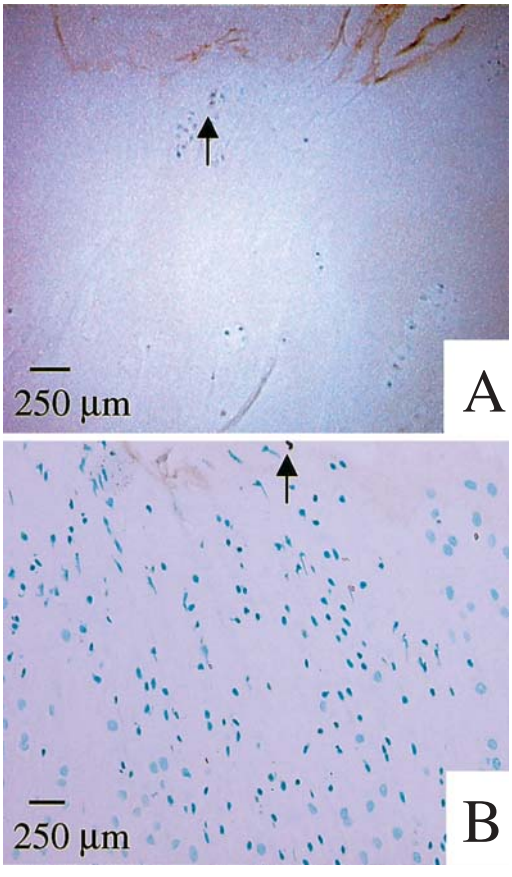

Fig. 7. TUNEL staining of human and rabbit cartilage after electroporation. **A** Human grade III cartilage tissue. **B** Needle penetration site of rabbit cartilage. The number of TUNEL-positive cells (*arrow*) was very low, and there was no apparent increase around needle penetration site

the number of cells that express transgene with the progress of degeneration grade, which seems to be favorable for a clinical application of EP to treat damaged cartilage.

In vivo gene transfer into articular cartilage, especially into the middle and deep layers of cartilage tissue, is not usually accomplished. Tomita et al.[19,20] have successfully performed in vivo direct gene transfer into rabbit articular cartilage using HVJ-liposomes, and about 30% of the cells in the superficial layer were transfected. However, the exogenous gene was seldom detected in the deeper layer of cartilage. Ikeda et al.[7] also reported that the expression of a marker transgene was detected mainly in synovial tissues, and only in the cells located on the surface in articular cartilage by direct in vivo injection of adenovirus vector in the knee joint of guinea pigs. These results indicate that it is not easy to deliver exogenous genes to deeper layers of articular cartilage. Gene transfer into thicker articular cartilage, such as that of humans, is likely to be still more difficult. There are several possible reasons for the low level of transfection efficiency into articular cartilage, e.g., the presence of a rich extracellular matrix, and the masking of receptors for viral vectors. The extracellular matrix is mainly composed of collagens and acidic proteoglycans, and is reported to have a mean pore size of 6 nm.[8] Cartilage usually accepts perfusion of low-molecular-weight molecules within the matrix. However, permeation of larger materials or molecules, such as liposomes and viral vectors, is physically disturbed. This indicates that the development of very small vectors or a different gene delivery system is needed for in vivo gene transfer to articular cartilage. Arai et al.[21] have reported that the use of a small vector, adeno-associated virus, could improve transfection efficiency into

articular cartilage tissue. Improvement of the direct gene delivery method by using collagen-based matrices[22] or EP could be another strategy.

EP, as used in the present study, can introduce foreign DNA into any kind of cell and tissue in a cell cycle-independent manner. It is one of the easily applicable nonviral methods, and uses plasmid DNA directly as a vector. One advantage of EP with a combination of naked plasmid DNA is its low toxicity, low immunogenicity, and biological safety, which are desirable for clinical applications. The efficiency of EP-mediated gene transfer is primarily regulated by physical parameters such as electric field strength, pulse duration, pulse frequency, wave form, and number of pulses. These parameters should be adjusted for different tissues and cells. Ohashi et al.[23] have reported that the optimal voltage of EP for the synovium was 150 V/0.7 cm, which was comparable to that reported by Aihara and Miyazaki[9] for skeletal muscle. We found that long pulses with a relatively low voltage (8 pulses, pulse length 50 ms, 2 Hz, 20–50 V) gave good gene transfer to articular cartilage, in combination with the newly developed superfine-tip electrodes. A further modification of the parameters could further improve the transfection efficiency of EP into articular cartilage.

At present, it is unclear how long the expression of transfected gene is maintained. Our present in vivo-simulating gene transfer and organ culture did not allow us to analyze how long the transgene expression persists. The direct application of EP to animal joints would help to answer this question. Nevertheless, several genes transfected by EP into various tissues have been reported to maintain expression for several weeks, and sometimes for several months.[15] Considering the stability of articular chondrocyte, which does not show active division within the matrix, it is possible that transgene expression persists for a relatively long period, although this expectation should be confirmed.

Our EP-mediated direct gene transfer method for degenerated cartilage allows gene transfer during joint operations or arthroscopy. What are the possible therapeutic genes for gene transfer to cartilage? Various growth factors, such as cartilage-derived morphogenetic protein (CDMP)-1, transforming growth factor (TGF)-β, and insulin-like growth factor (IGF)-1, are examples of potential candidate genes for enhancing cartilage matrix synthesis by the chondrocytes. Genes for anti-inflammatory cytokines, such as interleukin 10 (IL-10) and IL-1 receptor antagonist, may help to inhibit cartilage matrix breakdown. Genes for transcription factors that mediate chondrocyte differentiation may also be helpful. Alternatively, the transfer of genes that inhibit chondrocyte apoptosis may play a role, since chondrocyte apoptosis is closely related to the progress of articular cartilage degeneration. We are currently analyzing the therapeutic effects of some of the above-mentioned genes.

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