Expression of Transduced HSP70 Gene Protects Chondrocytes from Stress

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ABSTRACT. ***Objective.*** To investigate the efficacy of adenovirus vector mediated transduction of heat shock protein 70 (HSP70) gene to human chondrocyte-like cell (HCS-2/8) against heat stress.

Methods. Two adenovirus vectors that contain wild-type (AxSHEwt) or mutant-type (AxSHEmt) HSP70 gene, and that are regulated by SRα promoter, were constructed. The mutant-type lacks the area that expresses stress durability. One of the 2 adenovirus vectors was added to the cultures of human chondrocyte-like cells (HCS-2/8). Heat stress (48°C) was applied to the transduced cells for 2 h, and the efficacy of adenovirus vector mediated transduction of HSP70 gene against heat stress in the chondrocytes was investigated using alamar blue assay and MTT assay.

Results. Absorbance levels at 48°C were 300.3 ± 51.9 and 1.173 ± 0.011 in the controls, 278.5 ± 33.8 and 1.217 ± 0.018 in the AxSHEmt transduced cells, and 349 ± 14.7 and 1.371 ± 0.033 in the AxSHEwt transduced cells. The level in the AxSHEwt transduced cells was significantly higher than in the other 2 groups ($p < 0.05$). With 37°C treatment, no significant difference was observed.

Conclusion. Chondrocytes to which HSP70 gene was transduced had a significantly higher metabolic activity and viability under heat stress. (J Rheumatol 2001;28:330–5)

Key Indexing Terms:
ADENOVIRUS VECTOR | GENE THERAPY | OSTEOARTHRITIS
HEAT SHOCK PROTEIN | CHONDROCYTE

Changes of articular cartilage are an essential factor in the development of osteoarthritis (OA). When articular cartilage is degenerated, viscoelastic durability of the cartilage decreases, and friction increases[1,2]. These changes then seriously affect joint function. It is important to prevent cartilage degeneration at the initial stage of OA and also promote regeneration of the cartilage. Currently prescribed conservative therapies are intraarticular injection of hyaluronan or steroids[3-5], but these therapies are not sufficiently efficacious to suppress the progression of cartilage degeneration, and their efficacious period is also short.

On the other hand, advancement in molecular biology has been associated with progress in genetic engineering, and the pathology of several diseases has been clarified at the gene level and the findings utilized in clinical treatment[6-8]. In gene therapy, clinically effective gene can be administered as a remedy and the delivered gene is expressed in the target tissue for a long period of time. Gene therapy is also regarded as a new drug delivery system, and its usefulness has been examined for joint diseases[9-18]. The ideal target tissue in gene therapy would be synovial cells for rheumatoid arthritis and chondrocytes for OA.

Another advantage of gene therapy is the expression of substances difficult to administer from outside the cells. Heat shock proteins (HSP) are cellular proteins induced within cells under stress and that protect the cells from damage. HSP are difficult to administer, but can be delivered into the cells using gene transduction. HSP with a molecular weight of 70 kDa is called HSP70, and its induction is related to the strength of heat stress or pressure stress[19,20]. The volume of HSP70 induced is also reported to correspond to the severity of OA[21,22]. HSP70 protects cells against various stresses[23-25], thus it is expected to protect chondrocytes against stress and to be an important remedy for the prevention of OA and for the regeneration of articular cartilage.

In this study, HSP70 gene was delivered to cultured chondrocytes using an adenovirus vector, and was expressed at a high level in the cells. We examined its preventive effects against stresses on chondrocytes, and review the applicability of gene therapy using HSP70 in treatment for OA.

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MATERIALS AND METHODS

Chondrocyte culture. We used the human chondrocyte-like cell line HCS-2/8[26-28]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Gibco BRL Co., Gaithersburg, MD, USA), 60 µg/ml of kanamycin, and 0.292 mg/ml of L-glutamine (hereafter, this solution is referred to as 10% FBS/DMEM). The cells were cultured in a CO_2 incubator (5% CO_2 and 95% air) at 37°C, and the culture medium was changed twice a week.

Adenovirus vector. We used adenovirus type-5 based recombinant virus vector, which lacks E1A, E1B, and E3 regions from the adenovirus genome (36 kb); as a result it is replication-deficient.

Two adenovirus vectors were prepared as described[29,30]. In brief, one was AxSHEwt, harboring an expression unit of the wild-type of HSP70-E tag fusion gene under the control of SRα promoter; and the other was AxSHEmt, harboring a mutant-type expression unit of HSP70-E tag fusion gene that deletes the codons 438–618, which are required for peptide binding under the control of SRα promoter (Figure 1). Recombinant virus clones expressing the wild-type HSP70 gene or mutant-type HSP70 gene under the control of SRα promoter were isolated, propagated, and titrated as described[31].

Gene delivery. HCS-2/8 cells were suspended into 10% FBS/DMEM and seeded in 75 cm^2 plastic flasks at a density of 5×10^6 cells and cultured under standard conditions (37°C, 95% humidified air, 5% CO_2). On the following day, culture medium was removed, and each adenovirus vector diluted into 600 µl of 10% FBS/DMEM at a density of 2.5×10^9 plaque forming units (pfu) was added to the cells to achieve multiplicity of infection (MOI) = 500 pfu/cell. The plate was kept in the CO_2 incubator (5% CO_2, 95% air) at 37°C for 1 h for gene delivery into the cells, and then 10% FBS/DMEM was added to the culture medium.

Stress loading. Stress was applied using heat. Two days after transduction, the cells were suspended into 10% FBS/DMEM and seeded in 96 well plastic plates at a density of 2×10^4 cells, and cultured under standard conditions (37°C, 95% humidified air, 5% CO_2). On the following day, heat stress was applied. The culture dish was carefully sealed and placed in a circulatory hot water bath. Water temperature was increased to 48°C and maintained at that level for 2 h; the dish was removed from the bath, then 100 µl of 10% FBS/DMEM was added to the plate, and it was cultured in the CO_2 incubator (5% CO_2, 95% air) before being assayed. Control cells were kept in the water bath at 37°C for 2 h.

Alamar blue assay. At 24 h after the heat stress loading, 10 µl of alamar blue (AccuMed International Inc., Chicago, IL, USA) was added to the culture medium and cultured at 37°C for 12 h. The study waited an additional 24 h because in our preliminary experiments cytoprotective effects became comparable after 24 h. Optical densities were examined spectrophotometrically at 570 nm and 595 nm, and the reduced alamar blue volume was measured.

MTT assay. At 24 h after the heat stress loading, 10 µl of MTT (5 mg/ml) was added to the culture medium, and cultured at 37°C for 4 h. Optical density (OD) at 570 nm and 630 nm was measured spectrophotometrically.

Western blotting. AxSHEwt transduced and AxSHEmt transduced cells were harvested at 2 days after the transduction, washed twice with PBS, and dissolved by sonication in lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% DOC, 0.1% SDS]. Five micrograms of protein was fractionated on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under Laemmeli's condition[32] and transferred to PVDF membrane (Immobilon P, Millipore, Bedford, MA, USA). HSP70-E tag fusion protein was detected by probing the blot with anti-E tag monoclonal antibody (Pharmacia Biotec, Upsala, Sweden). The immunocomplex was detected as described[33].

Immunofluorescence. Two days after transduction, AxSHEwt transduced cells were washed with PBS, fixed in ice cold ethanol/acetate (95:5) for 15 min, and rinsed with Tris buffered saline 0.1% Triton X-100 (T-TBS). Nonspecific antibody was blocked by 1% skim milk for 1 h, rinsed with T-TBS, and incubated with the anti-E tag Mab for 1 h. Slides were washed with T-TBS, incubated with ExtrAvidin-fluorescein isothiocyanate for 30 min, washed again with T-TBS, mounted, and photographed.

RESULTS

Evaluation of HSP70 gene transduction by alamar blue assay. With 48°C treatment, OD at 570 nm (Abs570) and 595 nm (Abs595) was 300.3 ± 51.9 in the controls (cells without gene transduction), 278.5 ± 33.8 in AxSHEmt transduced cells, and 349 ± 14.7 in AxSHEwt transduced cells

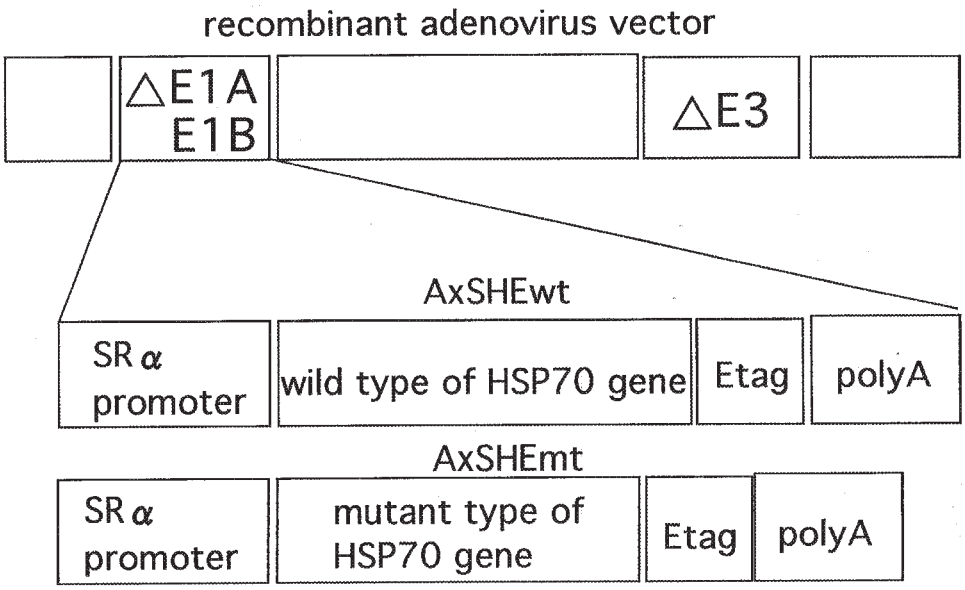

Figure 1. AxSHEwt harboring wild-type HSP70 gene and AxSHEmt harboring mutant-type HSP70 gene under the control of SRα promoter were constructed as illustrated.

(Figure 2). Absorbance in the AxSHEwt transduced cells was significantly higher than in the other 2 groups (n = 10 each; $p < 0.05$ by t test).

With 37°C treatment, Abs570-Abs595 was 477.5 ± 12.5 in the controls, 458.6 ± 19.8 in AxSHEmt transduced cells, and 470 ± 9.9 in AxSHEwt transduced cells (Figure 3). No significant difference was observed (n = 10 each).

Evaluation of HSP70 gene transduction by MTT assay. With 48°C treatment, OD at 570 nm (Abs570) and 630 nm (Abs630) was 1.173 ± 0.011 in the controls, 1.217 ± 0.018 in the AxSHEmt transduced cells, and 1.371 ± 0.033 in the AxSHEwt transduced cells (Figure 4). Absorbance in the AxSHEwt transduced cells was significantly higher than in the other 2 groups (n = 3 each; $p < 0.05$, t test).

With 37°C treatment, Abs570-Abs630 was 1.288 ± 0.013 in controls, 1.267 ± 0.055 in the AxSHEmt transduced cells, and 1.335 ± 0.067 in the AxSHEwt transduced cells (n = 3 each, Figure 5). No significant difference was observed.

Transduced HSP70 gene expression. We detected the protein product of expressed gene after the transduction using anti-E tag Mab, which recognizes immuno-tag fused to the C-terminal of HSP70. The tagged HSP70 protein was detected in both the AxSHEwt and AxSHEmt transduced cells, but not in the control cells (Figure 6).

In addition, HSP70 expression was confirmed by immunofluorescence. All the cells (100%) that received AxSHEwt transduction were stained positive (Figure 7A).

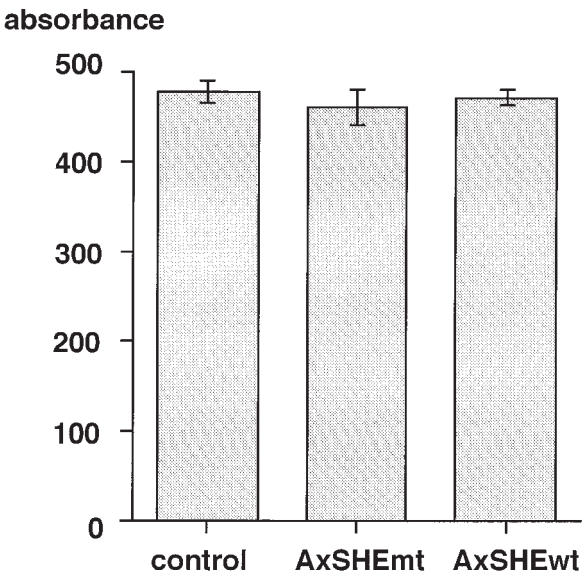

Figure 3. Alamar blue assay. When the cells were treated at 37°C, optical density Abs570-Abs595 was 477.5 ± 12.5 in controls, 458.6 ± 19.8 in AxSHEmt transduced cells and 470 ± 9.9 in AxSHEwt transduced cells. No significant group difference was observed (n = 10 each).

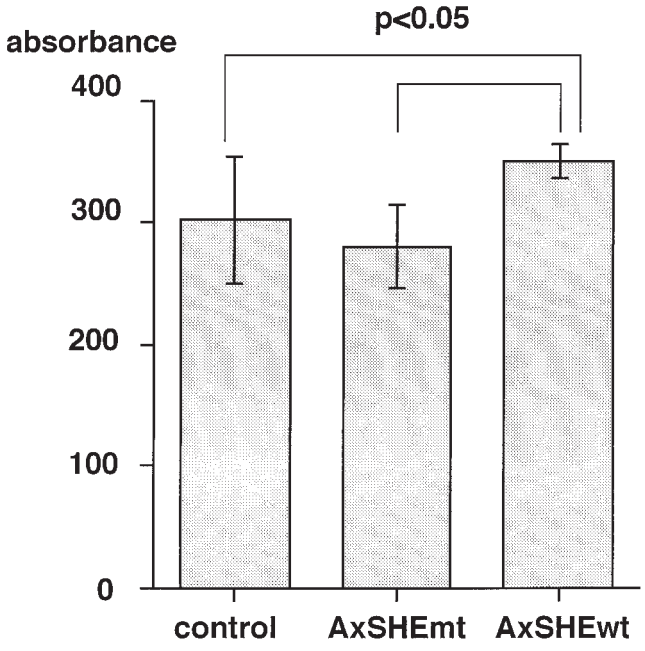

Figure 2. The alamar blue assay. When the cells were treated at 48°C, optical density Abs570-Abs595 was 300.3 ± 51.9 in the controls, 278.5 ± 33.8 in the AxSHEmt transduced cells and 349 ± 14.7 in the AxSHEwt transduced cells. Absorbance in the AxSHEwt transduced cells was significantly higher than in the other 2 groups (n = 10 each).

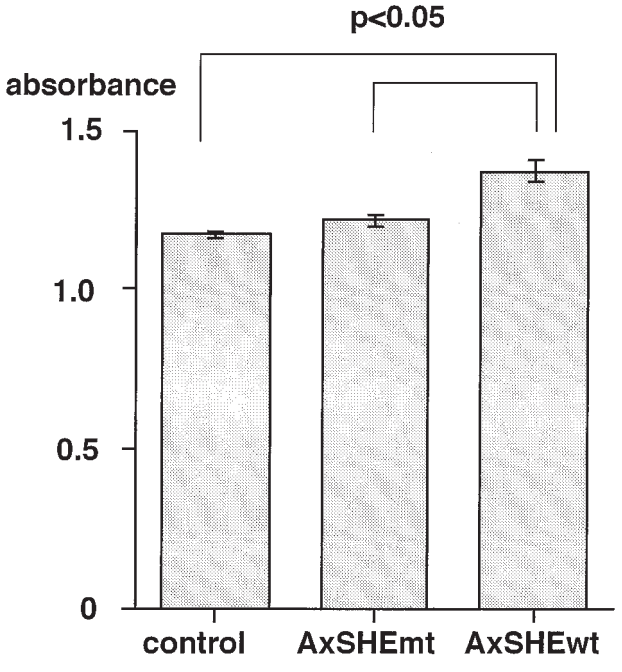

Figure 4. MTT assay. When the cells were treated at 48°C, optical density Abs570-Abs630 was 1.173 ± 0.011 in controls (cells without gene transduction), 1.217 ± 0.018 in AxSHEmt transduced cells, and 1.371 ± 0.033 in AxSHEwt transduced cells. Absorbance in the AxSHEwt transduced cells was significantly higher than in the other 2 groups (n = 3 each; $p < 0.05$, t test).

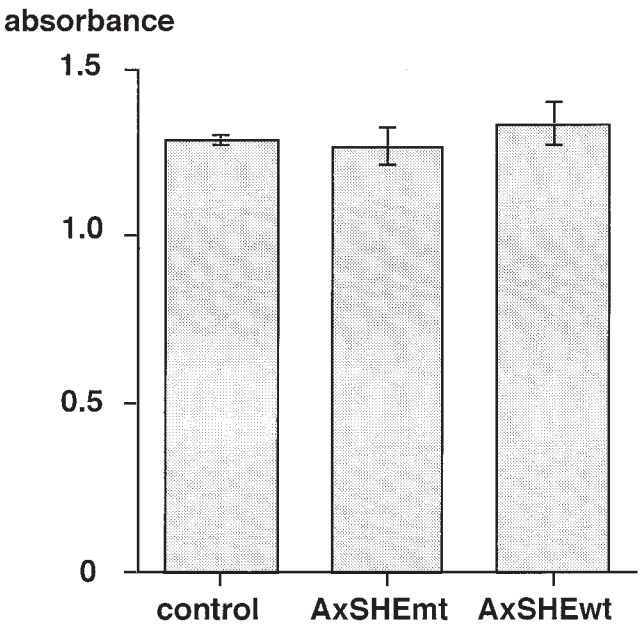

Figure 5. MTT assay. When the cells were treated at 37°C, optical density Abs570-Abs630 was 1.288 ± 0.013 in controls, 1.267 ± 0.055 in AxSHEmt transduced cells, and 1.335 ± 0.067 in AxSHEwt transduced cells. No significant difference was observed (n = 3 each).

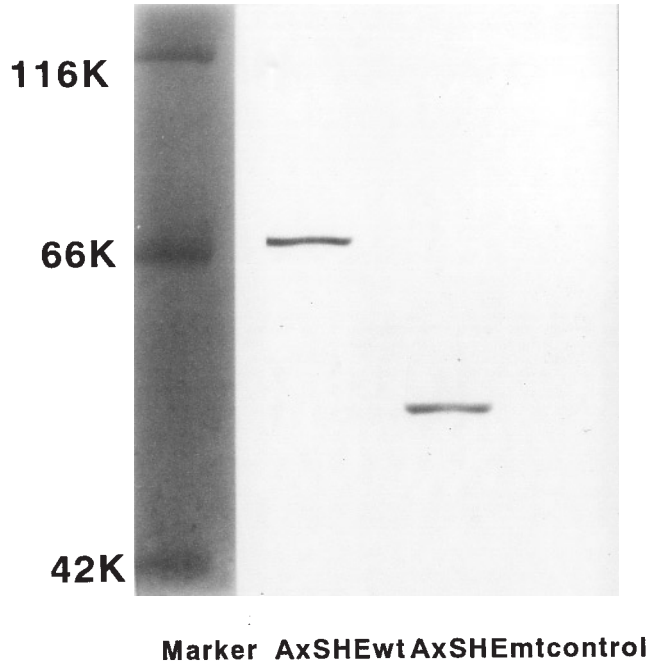

Figure 6. Western blot analysis confirmed the expression of transduced HSP70 wild-type and mutant-type genes.

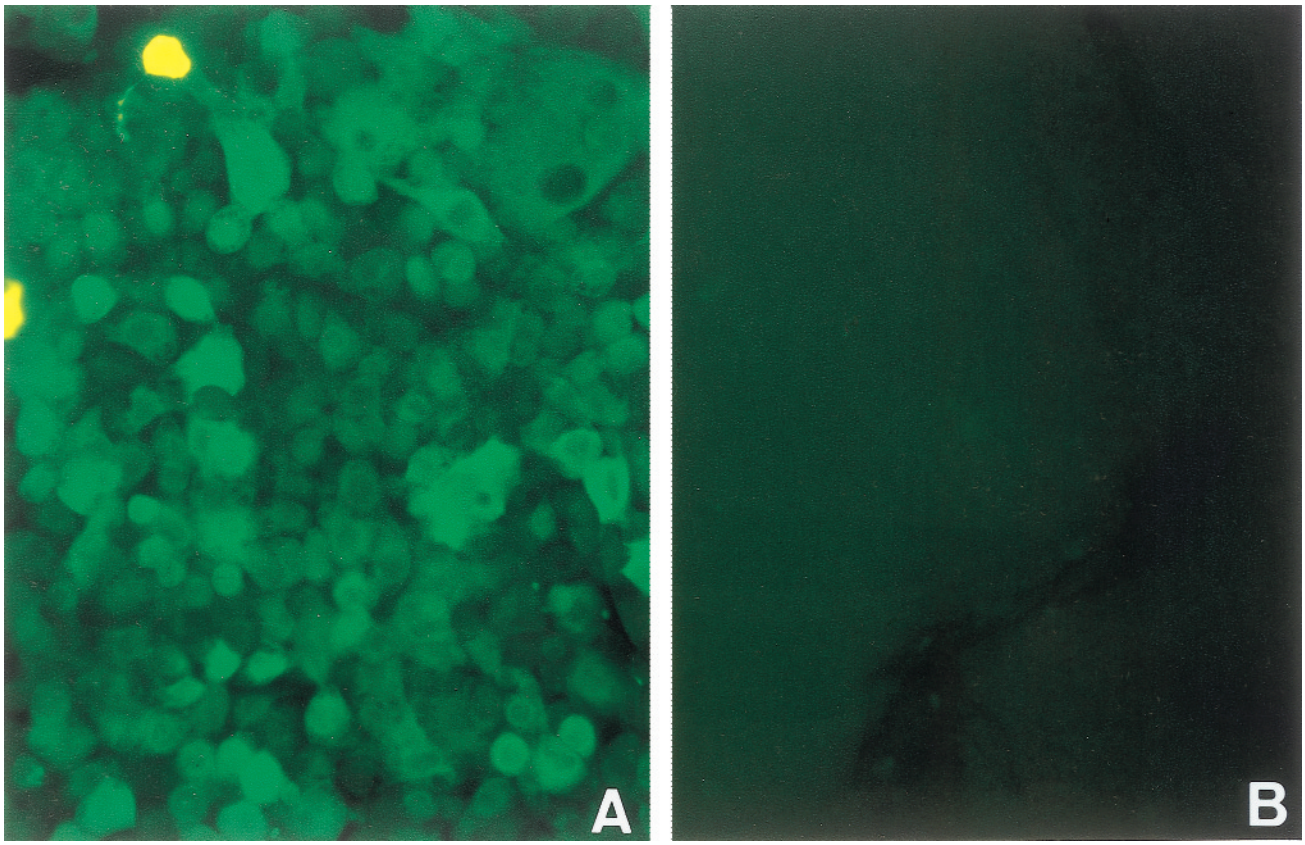

Figure 7. A. Immunofluorescence confirmed expression of transduced HSP70 gene in all cells (100%). B. Negative control cells.

The control cells without treatment were stained negative (Figure 7B).

DISCUSSION

HSP is a protein family that is induced within cells when various stresses are applied, and it is presumed to act as a molecular chaperone that plays some role in regulating normal proteins. HSP70 is one of the most important HSP. Its molecular weight is 70 kDa, and it is thought to protect protein structures against possible damage due to various kinds of environmental stresses. After treatment with mild heat stress (42°C), fibroblasts acquire resistance against lethal heat stress (45°C)[34]. On the other hand, intracellular injection of anti-HSP70 antibody reduces heat resistance[35]. Treatment of cardiac muscle cells with mild heat stress (42°C) enhanced postischemic ventricular recovery[36]. High expression of HSP70 in cardiac muscle cells protects the cells against apoptosis as well as against hypoxic stress[37]. Thus HSP70 has been thought to protect cells not only against heat stress but also against other stresses such as hypoxia, ischemic changes, and apoptosis.

In our study, the efficacy of HSP70 gene transduction was investigated using HCS-2/8 cells. These cells maintain their original phenotype in a long culture period. They produce collagen type II, IX, and XI and cartilage proteoglycans (aggrecan); respond to various vitamins and growth factors; and express protooncogenes similar to those of normal chondrocytes[26–28]. Based on these findings, the HCS-2/8 cell line is thought to be a good model of normal human chondrocytes. Because expression levels of type II collagen and proteoglycan mRNA do not alter after the transduction of HSP70 gene using an adenovirus vector[9], the cells are also thought to maintain chondrocyte phenotype following transduction. In our unpublished studies, we examined the level of heat stress lethal to HCS-2/8 in the range of 37~50°C using alamar blue assay and MTT assay, and observed the appearance of cytotoxicity at 48°C. Therefore, the lethal heat stress in the present study was set at 48°C.

In OA, chondrocytes receive physical stresses such as unphysiologically high weight loading and high temperature. In the joint fluid and cartilage tissues of OA, levels of catabolic cytokines such as interleukin 1 increase. These stresses and cytokines are known to accelerate HSP70 production in chondrocytes[20,38]. We have studied HSP70 expression in chondrocytes obtained from patients with OA and in cultured cell lines. Results showed that in OA chondrocytes HSP70 expression increased along with the increase of disease severity, and HSP70 expression was most readily observed in areas that receive great stresses[21,22]. These findings suggest that HSP70 expressed in OA chondrocytes would protect the cells against excessive stresses. In the present study, absorbance levels in the cells treated at 37°C did not change significantly between the 3 groups. This shows that HSP70 gene delivery using adenovirus vector is not associated with cytotoxicity. With alamar blue assay, absorbance at 48°C was 300.3 ± 51.9 in the controls (cells without gene transduction), 278.5 ± 33.8 in the AxSHEmt transduced cells, and 349 ± 14.7 in AxSHEwt transduced cells. With the MTT assay, absorbance at 48°C was 1.173 ± 0.011 in controls, 1.217 ± 0.018 in AxSHEmt transduced cells, and 1.371 ± 0.033 in AxSHEwt transduced cells. In all 3 groups, absorbance levels decreased from those at 37°C, and this shows that 48°C heat stress induced cytotoxicity. In the AxSHEwt transduced cells, the absorbance level was significantly higher than in the other 2 groups. This shows that AxSHEwt transduced cells had significantly higher metabolic activity and viability. Therefore, if a sufficiently large volume of HSP70 were expressed in chondrocytes of early OA, it would protect the cells against stresses and also prevent OA progression.

If gene products that act in both autocrine and paracrine fashion are delivered to chondrocytes in OA, there could be benefits not only on transduced chondrocytes but also nontransduced chondrocytes. However, HSP70 is a cellular protein, and it is not expected to have autocrine or paracrine actions. For its application, it has to be expressed within chondrocytes. Therefore, HSP70 transduction would have beneficial effects only when it is transduced to chondrocytes. In future studies, efficacy of HSP70 gene delivered to chondrocytes of animal models of OA will be investigated.

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