Anti-apoptotic Bcl-2 gene transfection of human articular chondrocytes protects against nitric oxide-induced apoptosis

S. Surendran, S. H. Kim, B. K. Jee, S. H. Ahn, P. Gopinathan, C. W. Han

From the Catholic University of Korea, Daejeon, Korea

We stably transfected early passage chondrocytes with an anti-apoptotic Bcl-2 gene in vitro using a retrovirus vector. Samples of articular cartilage were obtained from 11 patients with a mean age of 69 years (61 to 75) who were undergoing total knee replacement for osteoarthritis. The Bcl-2-gene-transfected chondrocytes were compared with non-transfected and lac-Z-gene-transfected chondrocytes, both of which were used as controls. All three groups of cultured chondrocytes were incubated with nitric oxide (NO) for ten days. Using the Trypan Blue exclusion assay, an enzyme-linked immunosorbent assay and flow cytometric analysis, we found that the number of apoptotic chondrocytes was significantly higher in the non-transfected and lac-Z-transfected groups than in the Bcl-2-transfected group (p < 0.05). The Bcl-2-transfected chondrocytes were protected from NO-induced impairment of proteoglycan synthesis.

We conclude that NO-induced chondrocyte death involves a mechanism which appears to be subject to regulation by an anti-apoptotic Bcl-2 gene. Therefore, Bcl-2 gene therapy may prove to be of therapeutic value in protecting human articular chondrocytes.

Degenerative disease of articular cartilage is characterised by loss of tissue cellularity and an alteration in the composition and organisation of the extracellular matrix. Studies have shown that apoptotic chondrocyte death plays a significant role in the pathogenesis of osteoarthritis.1,2 Blanco et al3 suggested that nitric oxide (NO) was the primary inducer of apoptosis in human articular chondrocytes. It has been found to damage the DNA strands and trigger apoptosis in normal human articular chondrocytes.3,4

Apoptosis is a biological process which is controlled by the expression of specific genes that are conserved in organisms from nematodes to mammals. Of these, the Bcl-2 gene family has been shown to play an important role.5 The protein encoded by the Bcl-2 gene has been implicated in the prolongation of cell survival by blocking apoptosis and necrosis.6,7 Feng et al8 found that Bcl-2 was an important factor for the survival of articular chondrocytes. Gene-transfer studies in a variety of mammalian cells have demonstrated the cytoprotective action of elevated Bcl-2 protein levels in these cells.9,11 These observations suggest that overexpression of the Bcl-2 gene in human articular chondrocytes may prevent their apoptosis in osteoarthritis. Although many studies have shown the association between chondrocyte apoptosis and levels of expression of the Bcl-2 gene,8,12,13 there are none which describe the effects of Bcl-2 gene transfection of human articular chondrocytes in the prevention of NO-mediated chondrocyte apoptosis in osteoarthritis.

Our aim was to evaluate whether retrovirus-mediated transfer of the anti-apoptotic Bcl-2 gene to human articular chondrocytes conferred a cytoprotective effect against NO-induced chondrocyte apoptosis in vitro.

Materials and Methods
The research protocol was reviewed and approved by the Ethical Care Committee at our hospital. All the patients gave informed consent.

Cartilage sampling. Articular cartilage was obtained from 11 patients, nine women and two men, with a mean age of 69 years (61 to 75), who were undergoing total knee replacement for osteoarthritis. Samples of articular cartilage were obtained from the non-weight-bearing upper lateral quadrant of the anterior aspect of the lateral femoral condyle using a scalpel by the same senior surgeon (CWH). Separate sets of experiments were carried out on chondrocytes obtained from each patient. Chondrocyte isolation and culture. The specimens of cartilage were finely minced, rinsed
with phosphate-buffered saline (PBS) three times and sequentially digested with 0.1% collagenase (GibcoBRL, Grand Island, New York) and deoxyribonuclease (0.1 mg/ml DNAse; Sigma, St. Louis, Missouri) at 37°C for three hours. The separated cells were then filtered through a 25 µm nylon filter and washed three times with PBS. The chondrocytes were seeded on to standard tissue-culture flasks (25 cm²) and cultivated in Ham's F-12 media, consisting of 10% bovine serum (GibcoBRL), Heps buffers (10 mM; Sigma), gentamicin sulphate (50 µg/ml; Gibco-BRL) and fungizone (2 µg/ml; GibcoBRL).

**Transfection of human chondrocytes with the Bcl-2 gene using a retrovirus.** The first passage of cultured chondrocytes was grown to 70% confluence in T-25 flasks (Nunc, Roskilde, Denmark) at 37°C under 5% CO₂. The cells were transfected by adding 10 µl of a suspension of a recombinant retrovirus containing the human Bcl-2, or lac-Z gene, in 1 ml of Ham’s F-12 media with 10% fetal bovine serum (FBS) for 24 hours in the presence of 8 µg/ml of polybrene. Recombinant retrovirus was supplied by the Department of Molecular Genetics and Biochemistry, University of Pittsburgh. The cells were then washed in PBS and transferred to Ham’s F-12 media containing 10% FBS and incubated at 37°C in 5% CO₂ for 72 hours. Transfected cells were then selected in a medium containing 0.5 mg/ml of G418 (AG Scientific Inc., San Diego, California). The lac-Z gene is used as a simple ‘reporter’ gene, separated from its natural promoter. Non-transfected and lac-Z-transfected chondrocytes were used as a pure control and empty vector control group, respectively.

**Analysis of Bcl-2 expression by immunocytochemistry.** In order to evaluate the transfection of the Bcl-2 gene, we performed immunocytochemical analysis using Bcl-2 antibody (1:200; Pharmingen, Los Angeles, California). Chondrocyte cells (5 x 10⁵) were plated on glass slides coated with Aptex (Sigma), treated with absolute methanol containing 0.3%H₂O₂ at room temperature for ten minutes, and incubated with the anti-Bcl-2 antibody at 4°C overnight. The slides were reacted with secondary antibody (Pierce Biotechnology, Rockford, Illinois) at room temperature for 30 minutes, and incubated with streptavidin-linked horseradish peroxidase conjugate, chromagen and tetramethylbenzidine at 405 nm absorption using a microplate autoreader (Molecular Devices, Sunnyvale, California). The results were expressed as an enrichment factor which represents the ratio of the optical densities for the control and experimental groups.

**Flow cytometry analysis.** Chondrocytes were detached from the culture flask using 1 ml of 0.25% trypsin and 0.02% ethylene diamine tetra acetic acid (EDTA) and incubated for three minutes at 37°C. The solution was centrifuged at 1300 rpm for ten minutes and washed with PBS three times. The supernatant fluid was removed and 5 µl of Annexin-V-fluorescein isothiocyanate (Nexins Research, Hoeven, The Netherlands) were added to each of the samples. After centrifugation, they were resuspended in 100 µl of binding buffer (10 mM Hepes/NaOH pH 7.4, 140 nM NaCl, 5 mM CaCl₂) and 1.0 µl of propidium iodide (1.0 mg/ml) stock solution (Molecular Probes, Leiden, The Netherlands) was added to each of the samples. The cells were incubated for 30 minutes at 37°C in the dark. Flow cytometry was performed for each sample on a Becton-Dickinson FACScan (Becton Dickinson, San Jose, California) immediately after staining.

**Assessment of proteoglycan synthesis by chondrocytes.** Incorporation of 35S by chondrocytes has been established as a measure of the synthesis of sulphated glycosaminogly-
cans, and therefore of proteoglycan. We placed $1 \times 10^5$ chondrocytes from each group in a six-well tissue plate and incubated this in a medium containing DMEM + 10% FBS and SNP 500 µM (NO donor) at 37°C with 5% CO₂ for ten days. Incubated cells were washed with PBS and provided with 0.5 ml of fresh Newman-Tytell serum-free medium (GibcoBRL), containing S-sulphate (final concentration 20 µCi/ml). After a period of seven hours, the labeling media were removed, and a cell layer from each well was extracted by shaking at 4°C for 48 hours by addition of 400 µl of PD-10 buffer (guanidine hydrochloride 4 ml/l, pH 7.0). For quantitative evaluation of S-sulphate in cell extracts and media, labelled proteoglycans and aliquots (200 µl) of the stored extracts and media, respectively, were eluted on Sephadex G-25M in PD 10 columns (Pharmacia Biotech, Uppsala, Sweden) under dissociation conditions. Fractions of 1 ml were then collected in scintillation vials, mixed with 7 ml of scintillation mixture (Ultima Gold; Packard Mariden, Connecticut) and counted in a liquid scintillation counter (Packard = 1990 TR). For conditioned media, 10 µl of aliquots were mixed with 7 ml of scintillation mixture and counted in a scintillation counter.

**Statistical analysis.** All the data were expressed as the mean ± SEM. Three groups were compared statistically using the Kruskal-Wallis H test and the Student-Newman-Keuls test. A p-value < 0.05 was considered to indicate significance.
Results

Retrovirus-mediated Bcl-2 gene transfer into human articular chondrocytes. We performed immunocytochemical staining of the chondrocytes to demonstrate directly retrovirus-mediated Bcl-2 gene transfer into cultured chondrocytes. Chondrocytes transfected with the Bcl-2 gene showed red staining of the cytoplasm and blue of the nucleus (Fig. 1a), whereas the control group showed no staining (Fig. 1b).

Assessment of cell viability based on the Trypan Blue exclusion assay. Trypan Blue exclusion is an assay of cell viability based on the exclusion of the dye by live cells and on blue staining by dead cells. In the presence of NO, the non-transfected and lac-Z-transfected chondrocytes showed a significant reduction in the number of viable cells. The percentage viability for the pure control group was 8.4% (± 5.3 SEM), for the empty vector control group 4.6% (± 3.9 SEM) and for the Bcl-2-transfected chondrocytes 45.7% (± 7.4 SEM), respectively (p < 0.01; Fig. 2).

DNA fragmentation assessment using nucleosome ELISA. This is based on a quantitative sandwich enzyme immunoassay which uses antibodies against DNA and histones, and determines mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. DNA fragmentation occurred in all three groups, but the amount differed. In non-transfected chondrocytes it was found to be 1.92 (± 0.03 SEM) and in lac-Z-transfected chondrocytes 1.94 (± 0.02 SEM), which was significantly higher than that in the Bcl-2-transfected chondrocytes 1.5 (± 0.004 SEM); p < 0.05; Fig. 3).

Chondrocyte apoptosis assessment using flow cytometry. Flow cytometry is based on the detection of phosphatidyl serine (PS) residues on the outer aspect of the cell membrane by Annexin-V. For the interpretation of the results, the lower left quadrant represents viable cells, the lower right quadrant chondrocytes in an early apoptotic state, the upper right quadrant chondrocytes in the late apoptotic state and the upper left quadrant chondrocyte necrosis. Flow cytometry analysis showed that the presence of apoptotic chondrocytes was significantly higher in the non-transfected (Fig. 4a) and lac-Z-transfected chondrocytes (Fig. 4b) compared with the Bcl-2-transfected group (Fig. 4c).

Functional assessment of chondrocytes measured by proteoglycan synthesis. The amount of sulphated proteoglycans produced by non-transfected chondrocytes was 100% (± 11.2 SEM) and lac-Z-transfected chondrocytes 67.6% (± 13.1 SEM). These were found to be significantly lower than that produced by Bcl-2-transfected chondrocytes 386.2% (± 14.5 SEM; p < 0.01; Fig. 5). This showed that Bcl-2 transfection allowed for a greater degree of preservation of synthetic function in the chondrocytes studied.
NO has been implicated as one of the main causes of chondrocyte apoptosis.\textsuperscript{5,16,17} Chondrocytes appear to be the major source of NO within the joint. Interleukin-1 and tumour necrosis factor-\textalpha{}, the major pro-inflammatory cytokines implicated in osteoarthritis, stimulate NO synthesis in articular chondrocytes.\textsuperscript{16,18} Even in the absence of any stimuli, cultures of osteoarthritic cartilage and synovial membrane from patients undergoing joint replacement have been found to produce NO spontaneously.\textsuperscript{19} The effects of NO on chondrocytes include inhibition of collagen and proteoglycan synthesis, modulation of metalloproteinases, increased susceptibility to injury by other oxidants, inhibition of B1-integrin-dependent signalling and apoptosis.\textsuperscript{19} Although many mechanisms for NO-mediated chondrocyte apoptosis have been postulated,\textsuperscript{17,20} a proven mechanism has not been demonstrated.

Bcl-2 is a cellular protein belonging to a family of gene products which regulate apoptosis, either as death antagonists (Bcl-2, Bcl-X\textsubscript{L}, Bcl-W, etc) or death agonists (Bax, Bak, Bad etc). The gene Bcl-2 derives its name from the initial discovery of this proto-oncogene at the chromosomal breakpoint of t(14;18)-bearing B-cell lymphomas, in which it is expressed in high levels leading to a survival advantage for the lymphoma cells.\textsuperscript{7} The Bcl-2 gene encodes a 25KDa protein, which can be detected intracellularly in the mitochondria, the perinuclear membrane and throughout the cytoplasm.\textsuperscript{5} Bcl-2 gene transfer has been shown to confer in vitro protection from apoptosis in isolated human pancreatic islets,\textsuperscript{9} and in vivo protection to injury to brain and liver cells in animal models.\textsuperscript{10,11,21,22} Although various theories have been proposed to explain the anti-apoptotic action of Bcl-2,\textsuperscript{5,7,23} none is fully convincing.

Although studies have demonstrated that agents such as insulin-like growth factor-1 (IGF-1), caspase inhibitors and dexamethasone may prevent chondrocyte apoptosis,\textsuperscript{24} Bcl-2 has not been demonstrated experimentally as an anti-apoptotic agent for human articular chondrocytes. Our study attempted to show that human articular chondrocytes could be efficiently transfected with a Bcl-2 gene using a viral vector, resulting in protection from NO-induced chondrocyte apoptosis in osteoarthritis. We found that Bcl-2 protected the transfected chondrocytes from NO-induced DNA fragmentation, as demonstrated by ELISA. To confirm further the cytoprotective action of Bcl-2 on chondrocyte apoptosis, we quantified apoptosis using the Trypan Blue exclusion assay and flow cytometry. The percentage viability of the Bcl-2 transfected chondrocytes was found to be significantly higher than in the control groups using the Trypan Blue assay. Annexin-V staining also showed significantly reduced apoptosis in Bcl-2-transfected chondrocytes when compared with the control. Furthermore, the NO-induced inhibition of proteoglycan synthesis was also found to be significantly reduced in Bcl-2-transfected chondrocytes. These findings suggest that Bcl-2 gene transfection of human articular chondrocytes provides protection against...
NO-induced apoptosis in osteoarthritis, in agreement with studies in other tissues.9,22

The main limitation of our study was the necessity of using a viral vector for gene transfer to articular chondrocytes. An in vivo study is required to evaluate the response of the human body to viral-mediated gene-transduced chondrocytes. Pre-existing immunity to the viral-mediated transgene products may reduce the efficiency of the gene transfer. The use of samples of articular cartilage from normal knees would have been more convincing, but it was very difficult to obtain approval from the Ethical Committee and consent from patients for the sampling of articular cartilage from normal knees.

Our study has shown that the transfection of human chondrocytes with an anti-apoptotic gene Bcl-2 provides protection against NO-induced chondrocyte apoptosis. The cytoprotective action of Bcl-2 gene transfer to human articular chondrocytes may give clinical promise for the prevention and treatment of osteoarthritis.

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References