Chondrocyte apoptosis in the regenerated articular cartilage after allogenic chondrocyte transplantation in the rabbit knee

J. H. Lee, 
K. V. B. Prakash, 
Y. H. Pengatteeri, 
S. E. Park, 
H. S. Koh, 
C. W. Han

From Histostem Research Center, Seoul, Korea

We attempted to repair full-thickness defects in the articular cartilage of the trochlear groove of the femur in 30 rabbit knee joints using allogenic cultured chondrocytes embedded in a collagen gel. The repaired tissues were examined at 2, 4, 8, 12 and 24 weeks after operation using histological and histochemical methods. The articular defect filling index measurement was derived from safranin-O stained sections. Apoptotic cellular fractions were derived from analysis of apoptosis in situ using TUNEL staining, and was confirmed using caspase-3 staining along with quantification of the total cellularity. The mean articular defect filling index decreased with time. After 24 weeks it was 0.7 (SD 0.10), which was significantly lower than the measurements obtained earlier (p < 0.01). The highest mean percentage of apoptotic cells were observed at 12 weeks, although the total cellularity decreased with time. Because apoptotic cell death may play a role in delamination after chondrocyte transplantation, anti-apoptotic gene therapy may protect transplanted chondrocytes from apoptosis.

The treatment of injuries to articular cartilage is one of the more intriguing orthopaedic challenges. Numerous procedures to repair the cartilage, including abrasion arthroplasty,1 subchondral drilling,2 osteochondral allografting,3 periosteal and perichondrial tissue grafting4,5 and chondrogenic cell transplantation,6 have undergone basic and clinical evaluation. Brittberg et al7 have described the results of implanting cultured autologous chondrocytes under a periosteal graft sutured to the defect. This technique offers a promising alternative to the majority of procedures currently in use. However, several complications, such as graft hypertrophy, arthrofibrosis, inadequate fixation to bone or adjacent cartilage,8 graft failure9 and a gradual decrease in the thickness of the grafted area,5 can occur after chondrocyte transplantation. Variations of the technique have been reported with good results.10 Failure of the graft after chondrocyte transplantation usually results from either delamination with variable degrees of filling defects, or from central degeneration of the repair tissue.9 Apoptotic cell death may contribute to delamination of the transplanted graft, thereby leading to degeneration of the repair tissue and progressive thinning of the regenerated cartilage. Isolated chondrocytes are susceptible to many types of damage during transplantation. The disruption of cell-matrix interactions during cell isolation can have major effects on phenotypic features such as gene regulation, cytoskeletal structure, differentiation, and aspects of control of cell growth.11 Apoptosis or programmed cell death is crucial to maintaining the appropriate number of cells and tissue organisation. This process also promotes other such phenomena induced by disruption of the interactions between cells and the extracellular matrix, a process termed anoikis.12 Furthermore, chondrocyte reactions to external stimuli can be modulated by changes in the extracellular matrix.13 Genetic modification of the chondrocytes may aid healing.14 After an isolated injury, chondrocytes may continue to experience additional stress because of the presence of proinflammatory cytokines, immune processes and other extracellular influences that can compromise the function of, or even kill, the transplanted cells. Apoptotic chondrocytes have been observed in cartilage during endochondral ossification,15 chondrogenesis,16 in the hypertrophic region of growth plates,17 in osteoarthritis (OA)18 or rheumatoid arthritis,19 and within articular cartilage damaged by impact.20

Because apoptosis of the chondrocytes transplanted into areas of cartilage defects may lead to failure of the graft or reduced filling of the defect, we elected to further assess the occurrence of apoptotic chondrocyte death in the areas of defect in the articular cartilage following chondrocyte transplantation in the knee of the rabbit.
Materials and Methods

The research protocol was reviewed and approved by the Animal Ethical Committee of our university.

We used 30, 12-week-old New Zealand White rabbits with a mean body weight of 2.5 kg (2.2 to 3.1). Slices of articular cartilage were taken from the knee, hip and shoulder joints of the animals by scalpel after aseptic preparation and were detached from adherent connective tissue before being cut into small slices. Chondrocytes were isolated from these slices by enzymatic digestion, as described elsewhere. Briefly, the slices were minced and washed three times with sterile 0.9% sodium chloride. They were then treated with 0.25% trypsin in sterile saline for 30 minutes, followed by 0.25% collagenase type II (Gibco BRL, Gaithersburg, Maryland) for six hours at 37°C in a culture tube. Chondrocytes were seeded at a density of 1.5 × 10^6 cells/ml. Then, 125 µl of the mixture was placed in a 20 mm diameter culture dish. The cell-collagen composites were incubated at 37°C for 30 minutes to allow the collagen to gel, and then overlaid with 2.0 ml of culture medium. Cell cultures were incubated in 5% CO₂/95% air at 37°C and were replaced with fresh Dulbecco’s Modified Eagles Medium (DMEM) (Gibco BRL) every three days.

Operative procedure. The chondrocytes were cultured in collagen gels (Celtrix, Santa Clara, California) containing the culture medium at a concentration of 2 × 10^6 cells/ml. Then, 125 µl of the mixture was placed in a 75 cm² T-flasks (Nunc, Kamstrupvej, Denmark) and allowed to grow until 50% confluence. After the isolation of chondrocytes with 0.25% trypsin, they were embedded in bovine type I collagen gels (Celtrix, Santa Clara, California) containing the culture medium at a concentration of 2 × 10^6 cells/ml. Then, 125 µl of the mixture was placed in a 20 mm diameter culture dish. The cell-collagen composites were incubated at 37°C for 30 minutes to allow the collagen to gel, and then overlaid with 2.0 ml of culture medium. Cell cultures were incubated in 5% CO₂/95% air at 37°C and were replaced with fresh Dulbecco’s Modified Eagles Medium (DMEM) (Gibco BRL) every three days.

Quantification of articular defect filling index. In order to harvest the specimens, we killed the rabbits at 2, 4, 8, 12 and 24 weeks after transplantation. Six distal femurs were each dissected after 2, 4, 8, 12 and 24 weeks, respectively, yielding a total of 30 specimens, which were then fixed with 10% buffered formalin for one week. They were then decalcified with decalcifying solution (Surgipath Medical Industries, Richmond, Illinois) for two days and embedded in paraffin. Sagittal sections, 5 µm thick, were obtained from the centre of each defect and each section was stained with safranin-O fast green to assess the proteoglycan content of the repaired tissue and to measure the articular defect filling index (ADFI), which is expressed as the ratio of the repaired articular area to the imaginary normal articular area, and was measured using histomorphometric analysis by Meta Imaging Series 4.6 (Universal Imaging Co., Downington, Pennsylvania). A, area of the repaired articular cartilage; B, area of the imaginary normal cartilage; ADFI = A/B.

In situ Detection of apoptosis. Sections of cartilage 5 µm thick were placed on to poly-L-lysine (Sigma-Aldrich, St Louis, Missouri) coated slides. Detection of apoptosis in situ was performed using the in situ cell death detection kit (Roche, Mannheim, Germany). Briefly, sections were deparaffinised with xylene, dehydrated in a graded series of ethanol, and incubated with proteinase K (20 µg/ml) (Bio-neer, Daejeon, Korea) for 25 minutes at 37°C. The speci-
mens were then incubated with 0.3% hydrogen peroxide/methanol for 10 minutes at room temperature. Slides were further incubated with permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for two minutes on ice. They were then treated with a reaction mixture of terminal deoxynucleotidyl transferase to incorporate digoxigenin-labelled nucleotides into DNA strand breaks. These slides were then incubated with anti-digoxigenin antibody coupled to horseradish peroxidase, followed by the application of diaminobenzidine chromagen substrate, which labels nuclei brown. Simultaneously, a standard indirect immunohistochemistry method was used to stain and detect caspase-3 activity, as described previously. Apoptotic cells in the cartilage were observed by high-power field microscopy (Nikon, Garden City, New York). The apoptotic index was expressed as the number of labelled nuclei per high-power field of the regenerated articular cartilage in an area 100 µm wide by 100 µm deep, measured from the articular surface at the centre of the defect. Two blinded observers (JHL, KVBP) calculated the apoptotic index independently, and their results were averaged. The total cellularity of each specimen was determined in a similar way by performing total cell counts per high power field on haematoxylin-counterstained sections.

Statistical analysis. All numerical data were expressed as the mean ± standard deviation (SD). The mean and SD of all data were compared between groups using the Kruskal-Wallis H test following Student-Newman-Keuls test for statistical analysis. Statistical significance was defined as p < 0.05.

Results
Nearly all samples evaluated at 2, 4 and 8 weeks had healed with some degree of elevation over the adjacent normal
cartilage (Fig. 2). The ADFI indexes obtained were 4.24 (SD 0.08), 3.24 (SD 0.15), 2.78 (SD 0.32), 1.8 (SD 0.32) and 0.7 (SD 0.1) at 2, 4, 8, 12 and 24 weeks after transplantation, respectively (Fig. 3). It thus decreased over time and at 24 weeks, which was significantly lower (Student-Newman-Keuls test, p < 0.01) than that measured in the other groups at earlier time points.

Total cellularity. In order to evaluate the sequential change in the number of cells in the transplanted chondrocytes prior to the determination of the apoptotic cell count, the total cellularity was assessed at the centre of the defect in the articular cartilage in an area 100 µm wide by 100 µm deep, as measured from the articular surface (Fig. 4a). The mean number of cells obtained in the normal cartilage at 2, 4, 8, 12 and 24 weeks after chondrocyte transplantation were 19.8 (SD 2.6), 21.2 (SD 2.3), 17.7 (SD 3.4), 15.3 (SD 2.8), 14.8 (SD 1.3), and 6.1 (SD 2.6), respectively (Fig. 4b), including the control. The total measurements of cellularity at two and four weeks were similar to that of the control group, but at 24 weeks was significantly lower than observed in the other groups (Student-Newman-Keuls test, p < 0.01). After 12 weeks, the total cellularity rapidly decreased. We hypothesised that one reason for this might be that apoptosis occurred after 12 weeks in the transplanted chondrocytes.

Apoptotic indices. In order to evaluate the degree of apoptosis in the chondrocytes after transplantation into the defect, we performed terminal deoxynucleotidyl transferase-mediated biotin-16-dUTP nick-end labelling (TUNEL) staining, which revealed a clear pattern of nuclear staining (Fig. 5). There was a distinct increase in labelling at the superficial layer of cartilage which faded in cells located in the deeper layers. Six samples of normal cartilage showed rare TUNEL-positive chondrocytes in the superficial zone (mean 2.35 SD 2.6%; 0 to 5.8). Detection of DNA fragmentation in the transplanted tissue within the defect revealed that apoptotic cells appeared at the articular surface at 2, 4, 8 and 24 weeks after transplantation, and progressed to a depth of at least 25 µm below the articular surface by 12 weeks, indicating that the cells present on the surface are more susceptible to apoptosis than those situated in the deeper layers. The mean percentages of apoptotic cells found in the sections from the defect were 2.4 (SD 2.6, mean value of control), 20.7 (SD 12.4), 10.6 (SD 9.6), 18.3 (SD 17.2), 76.7 (SD 10.3) and 30.1 (SD 17.3) in the controls 2, 4, 8, 12 and 24 weeks after transplantation, respectively (Fig. 6). The highest mean percentage of apoptotic cells was obtained 12 weeks after chondrocyte transplantation, and the number of apoptotic chondrocytes observed at 12 weeks after transplantation was significantly higher (Student-Newman-Keuls test, p < 0.01) than that observed at 24 weeks. The expression of caspase-3 was also highest at 12 weeks after allogenic chondrocyte transplantation, and again in the superficial zone (Fig. 7).

Discussion
Skeletal development requires intricate co-ordination of cellular growth, differentiation, apoptosis, remodelling of the extracellular matrix, and angiogenesis. During the for-
formation of endochondral bone, an avascular tissue (cartilage) is replaced by one of the most highly vascularised, bone. In our experiments, articular cartilage showed a marked reduction in both measurement of ADFI and total cellularity as time progressed after chondrocyte transplantation. One of the reasons for the reduced ADFI is that ossification begins with the invasion of the calcified hypertrophic cartilage by capillaries, which is accompanied by apoptosis of terminal hypertrophic chondrocytes, degradation of the cartilage matrix, and deposition of bone matrix by osteoblasts. Although ossification at the base of the graft is the main reason for the reduced ADFI observed at 24 weeks, delamination with variable degrees of filling of the defect, or central degeneration of the repair tissue after chondrocyte transplantation, might play an important role in the reduced ADFI.

Apoptosis has been identified as one of the mechanisms of cell death, and plays a particularly important role in controlling the number of cells as cells compete for a limited amount of survival factors. In our experiment we observed a reduction of cellularity of the superficial layer of the transplanted cells in the area of the defect in the articular cartilage. One of the reasons for this hypocellularity might be chondrocyte apoptosis at this site. We therefore investigated whether apoptotic cell death occurs after transplantation of chondrocytes to the defect.

Canine studies have shown that transplanted chondrocytes undergo a sequential pattern of healing, including stages of proliferation between zero and six weeks, transition between seven and 12 weeks, and remodelling between 12 weeks and at least three years. During the remodelling stage, the transplanted chondrocyte tissue integrates with the surrounding cartilage and subchondral bone, resulting in increased cross-links between matrix proteins and stabilisation of the collagen framework within the subchondral bone. During the remodelling phase of wound healing in the skin, scar tissue is heralded by a decrease in cellularity, and apoptosis is the mechanism responsible for cell removal during the transition from granulation tissue to scar. In a manner similar to the healing process in skin, we observed that during the remodelling phase, at 12 weeks after transplantation, the transplanted chondrocytes in the repaired articular cartilage showed the highest percentage of apoptosis (Figs 5 and 6). Therefore, we consider that chondro-
The percentage of apoptotic cells observed in the sections of cartilage in the control group at 2, 4, 8 and 24 weeks after transplantation ranged between 0% and 50%. In some sections we did not find apoptotic cells, and it was necessary to analyse several consecutive slides to obtain positive results. This indicates that apoptosis may not have been triggered simultaneously in all the cartilage chondrocytes, and that apoptotic involvement of the cartilage could be focal. Apoptosis can run its course very quickly, the process finishing within minutes to hours. The duration of the apoptosis of chondrocytes in cartilage is unknown.\(^{18}\) The measurement of apoptosis by TUNEL staining in degenerated articular cartilage is clearly prone to overestimation if not performed properly.\(^27\) In a recent study, counting of apoptotic cells was characterised morphologically by a condensed nucleus, rather than by relying on the TUNEL reaction. Interestingly, although the pattern of TUNEL staining in these experiments correlated with that of the distribution of the apoptotic cells, the percentage of TUNEL-positive cells was roughly ten times higher than that of apoptotic cells.\(^{28}\) However, we applied the same conditions in all our groups and consistently found that apoptosis occurred in 76.7% (SD 10.3) of cells at 12 weeks. This high number of apoptotic cells during the remodelling phase could reduce the total cellularity, induce accelerated cartilage degradation and result in degenerative changes in the cartilage by 24 weeks after transplantation. We also determined the extent of apoptosis in the cartilage filling the articular defect, using the TUNEL method and by estimating the expression of caspase-3, one of the key enzymes that mediates the final stage of the cell death by apoptosis.\(^{29}\) We found that expression of both caspase-3 and TUNEL was highest in the superficial zone 12 weeks after chondrocyte transplantation.

We did not follow the fate of the transplanted chondrocytes further in this study. Previous studies in rabbits using titrated thymidine-labelled chondrocytes have shown that \textit{in vitro} cultured chondrocytes were represented in repair tissue formed \textit{in vivo}.\(^{30}\) Therefore, we presume that the apoptotic cells observed in our study could have been the transplanted allogenic chondrocytes that were present in the defect in the articular cartilage. However, they may have also originated from the periosteum. Unfortunately, our experiment was not able to differentiate between these two sources of apoptotic cells. We recognise this as a limitation of our study. Although the TUNEL method is useful for identifying apoptotic cells, there may be conditions involving extensive DNA degradation, even selective to internucleosomal DNA, accompanying necrosis. There is a possibility that necrosis resulting from immune rejection of the transplanted area occurred, because the chondrocytes used in this experimental model were allogenic and potentially immunogenic. However, chondrocytes within a matrix usually have limited episodes of immune rejection.\(^{18}\) Another factor that may contribute to apoptosis is the occurrence of interaction between autologous periosteal cells and allogenic chondrocytes.

Apoptosis, a highly regulated process of cell death, is controlled through the expression of specific genes that are largely conserved in a wide variety of organisms, ranging from nematodes to mammals.\(^{31}\) The bcl-2 family comprises some of the most prominent anti-apoptotic genes. The protein encoded by the bcl-2 gene has pleiotropic anti-apoptotic effects, acting within both the mitochondria and the cytosol.\(^{32,33}\) Gene transfer of A20, another member of the anti-apoptotic bcl-2 family, inhibits cytokine-induced apoptosis and nuclear factor-κ-B activation in murine and human pancreatic islets.\(^{34}\) Our earlier study on transfection...
of human chondrocytes with an anti-apoptotic gene demonstrated that bcl-2 provided cytoprotective action to the articular chondrocytes. As our study demonstrates high rates of apoptosis in chondrocytes transplanted to articular cartilage defects, the use of anti-apoptotic gene therapy such as bcl-2 may provide cytoprotective effects via anti-apoptotic action, and may thus improve graft survival and articular defect filling. However, further studies are required in this regard.

In summary, we have shown that during the remodelling stage after chondrocyte transplantation there is a rapid, significant decrease in total cellularity, along with a concomitant significant increase in apoptosis, especially in the superficial layers. Therefore, apoptosis may be one of the factors responsible for the decrease in total cellularity of transplanted chondrocytes during the remodelling stage of chondrocyte transplantation.

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References