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Intercellular signaling as a cause of cell death in cyclically impacted cartilage explants

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Summary

Recently, *in vitro* cartilage studies have shown that impact loading can produce structural damage and osteoarthritis-like changes, including tissue swelling, collagen denaturation, and cell death.

Objective: This study was to determine whether a signal for cell death moves through the cartilage matrix, resulting in the spread of cell death over time from impacted to unimpacted regions.

Design: Cyclic impacts were applied to the 2 mm core of 4 mm cartilage discs. Post-impact culturing extended for 3, 6 or 21 days and occurred in one of two ways. In one, discs were cultured intact. In the second, cores were removed immediately after cessation of impact and cores and rings cultured separately. Cells in apoptosis and later stage necrosis were monitored using the TUNEL assay.

Results: The extent of cell death in impacted samples increased with increased duration of post-impact culturing. At the early time, the majority of cell death was located in the regions of direct impact whereas after extended culture, the extent of cell death was similar in the surrounding unimpacted regions and in the impacted core region. However, the physical separation of the impacted core from the surrounding, non-impacted ring regions immediately after impact, and prior to independent culture, kept the level of cell death in the surrounding ring close to control levels, even after 21 days of incubation.

Discussion: These findings indicate that soluble intercellular signalling is involved in the spreading of cell death through the cartilage matrix, and that its effects can be prevented by physical isolation of the surrounding ring from the impacted core. © 2001 OsteoArthritis Research Society International

Key words: Cartilage, Apoptosis, Mechanical damage, Osteoarthritis.

Introduction

A decrease in chondrocyte numbers, along with elevated water content, loss of proteoglycans, and the presence of denatured collagen has been documented in osteoarthritic cartilage[1–5]. Osteoarthritis (OA) is thought to be caused by mechanical damage to the cartilage matrix, and in *in vitro* studies, mechanical damage results in OA-like changes in the cartilage. Cyclic mechanical impacts on cartilage explants have been shown to cause damage to the cartilage matrix as detected by increased water[6], and denatured collagen[2]. Other consequences of cyclic mechanical impacts that cause matrix damage include cell death and a decrease in GAG content over time.[7,8] Studies have shown that there is an increase in cell death associated with increased duration of cyclic impacts[7]. Recent reports have indicated that *in situ* apoptosis is a cause of the reduced cell viability in osteoarthritis[9,10], but evidence supports the view that considerable necrosis results from the *in vitro* cyclic impact studies[7]. The presence of necrotic cells in *in vivo* OA, however, has been studied little, and may play a critical role in the early onset of the disease. Also, physiological cell death of chondrocytes within the cartilage matrix may be complex, differing at least morphologically from classical apoptosis[11]

Much research has been conducted as to what mediates cell death in the chondrocytes during OA. Evidence supports the idea that several independent pathways for apoptosis can occur in chondrocytes, one of which is mediated by the increased concentration of nitric oxide (NO) in osteoarthritic chondrocytes and the surrounding matrix[12]. Other possible initiators of cell death in osteoarthritic chondrocytes are cytokines, such as IL-1β, TNF-α[13], and Fas ligand[14].

In our previous studies, we focused on cell death at 2 days post-loading. In such experiments, cell death was localized to the core regions of the cartilage explants, which were directly impacted by the mechanical loading apparatus, with little incidence of cell death in the surrounding ring. No observations were made beyond 6 days. However, we reasoned that an intercellular signal may move through the cartilage matrix over time, leading to more expansive and less localized cell death in samples incubated for longer periods. In these experiments we tested the hypothesis that cell death is induced via an intercellular signaling

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mechanism from impacted regions to non-impacted regions, and can be prevented in non-impacted regions by physical isolation from impacted regions.

Methods

EXPLANTS

Cartilage explants were obtained from the articular surface of canine shoulders as 4 mm disks, using a 4 mm biopsy punch and a No. 10 surgical blade. Disks were of uniform thickness—about 0.7 mm. The two donor animals were 8–10 month-old, skeletally mature, Labrador retrievers from a breeding colony at the James A. Baker Institute for Animal Health, and the shoulders and hips of both animals were macroscopically normal at necropsy. Explants of cartilage from both animals were pooled together, and distributed at random among the different treatments. The explants were washed with Gey's balanced salt solution, and transferred to 24-well plates with serum-free Ham's F12 culture medium for incubation at 37°C, 95% humidity, and 5% CO_2 for up to 48 h before cyclical impacts. The culture medium was prepared by adding 2.5 ml Hepes buffer solution (1 M), 1.0 ml α-ketoglutarate (3 mg/mL), and 1.0 ml aqueous $CaCl_2$ (4.85 g/100 ml) to 100 ml Ham's F12. To this solution was added 0.2 ml gentamycin (10 mg/ml), 0.2 ml penicillin-streptomycin (10,000 U/ml Pen-G, 10,000 µg/ml streptomycin sulfate), and 0.4 ml fungizone (250 µ/ml). After filtering through a 0.22 µm filter, this medium was supplemented with 1.0 ml ITSCR+ (insulin (12.5 mg/20 l), transferrin (12.5 mg/20 l), selenous acid (12.5 µg/20 ml), BSA (2.5 g/20 ml), linoleic acid (10.7 mg/20 ml)), and kept at 4°C in the dark. Immediately before changing culture medium, the solution was further supplemented with 1.0 ml L-glutamine (30 mg/ml) and 1.0 ml ascorbic acid (50 mg/ml) per 100 ml of culture medium.

CYCLIC IMPACTS

Cyclic impact loads were applied to the 2 mm core of the 4 mm disk using a dynamic loading machine developed in our laboratory. Load control and data acquision were accomplished using Labview 4.1 software working on a Power Macintosh 8100/80 micro-computer equipped with NB-MIO-16L and NB-DMA-2800 data acquisition boards. The computer software regulated the air pressure incurred by a servovalve, which lifted the loading chamber into the stationary indentor, as described previously[6]. The cartilage disks were held in place by a stainless steel ring, and the loading chambers were filled with Gey's balanced salt solution during impacting. Sixteen explants were loaded for 20 min, 16 for 120 min, and an additional 16 explants served as the non-loaded controls. Impact stress reached 5 MPa and was applied at a maximum loading rate of 60 MPa/s. This regimen was repeated at 0.3 Hz, of which active loading lasted for 1.0 s per cycle.

INCUBATION AND EXPLANT TREATMENT

After impact loading, the cartilage disks were cultured in the serum-free Ham's F12 culture medium described above for incubation at 37°C, 95% humidity, and 5% CO_2 for 3 (12 discs), 6 (18 discs), and 21 (18 discs) days, with culture medium changed every third day. Six discs from the 6-day cultures and six disks from the 21-day cultures (duplicates of 0, 20, and 120 min impact times for each) had the directly impacted core regions removed with a 2 mm biopsy punch, within 5 min of the cessation of impacts. Control samples were disks with the cores kept intact.

Two samples from each treatment group were frozen in Gey's balanced salt solution until papain digestion and determination of glycosaminoglycan content by the dimethylmethylene blue assay[15] to estimate proteoglycan content. The remaining specimens were frozen in liquid nitrogen in optimal cutting temperature (OCT) compound, and stored at −70°C for staining with fluorescent nucleotides in the TUNEL assay.

In a similar experiment, explants were impacted for 120 min with the cores removed immediately after cessation of impacting. These samples, however, were cultured together with unimpacted whole cartilage disks for 21 days under the conditions previously described. The discarded culture media, as well as the Gey's solution used during impacting, were saved for later analysis of nitric oxide concentration. Cores and whole cartilage discs were frozen in OCT and stored at −70°C for the TUNEL assay.

TUNEL LABELING

The frozen samples were cut in 6 µm-thick cross-sections using a microtome cryostat at −35°C, and adhered to slides coated with chrome-aluminum gelatin. Four sections were adhered to each slide, and two slides were analysed per explant. One slide contained sections cut 1.25 mm from the circumference of the 4.0 mm disk and the second slide contained sections cut 1.75 mm from the circumference of the 4.0 mm cartilage disk. Cell death was detected by the Terminal dUTP Nicked-End Labeling (TUNEL) method which will identify cells in apoptosis and later stage necrosis[7]. Slides were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, 0.1 M phosphate-sodium, 0.15 M NaCl, pH 7.4) for 30 min, and rinsed with PBS. Samples were then washed in fresh PBS for another 30 min. The sections were permeabilized (0.1% Triton X-100 in 0.1% Sodium Citrate) on ice for 2 min. After being returned to room temperature, the cartilage samples were digested for 15 min in a Proteinase K solution (20 µg/ml PBS). Positive control slides were treated with DNase I (1 mg/ml), for 20 min at 37°C. Labeling was done using the Boehringer-Mannheim *in situ* Cell Death Detection Kit with fluorescein, by mixing 5 ml of terminal deoxynucleotidyl transferase (TdT) enzyme with 45 ml of fluorescein-labeled dUTP (per five cartilage sections) and incubating for 60 min at 37°C. Since the cell membranes were permeabilized during the TUNEL procedure, the total cell count was determined by a counterstain with a 40 mg/ml propidium iodide (PI) solution. Slides were then fixed in the antifade agent, gelvatol with diazabicyclooctane (DABCO), and stored at −70°C in the dark.

For examination of morphological features of our dead cells to determine the mode of cell death, our sections were labeled with the TUNEL procedure outlined above, followed by a counterstain with 0.2 M Hoechst dye (No. 33342) for 5 min. Slides were then fixed and stored as described above.

CELL DETECTION AND COUNTING

Fluorescence was determined using an Olympus I×70 confocal fluorescent microscope attached to a PC running

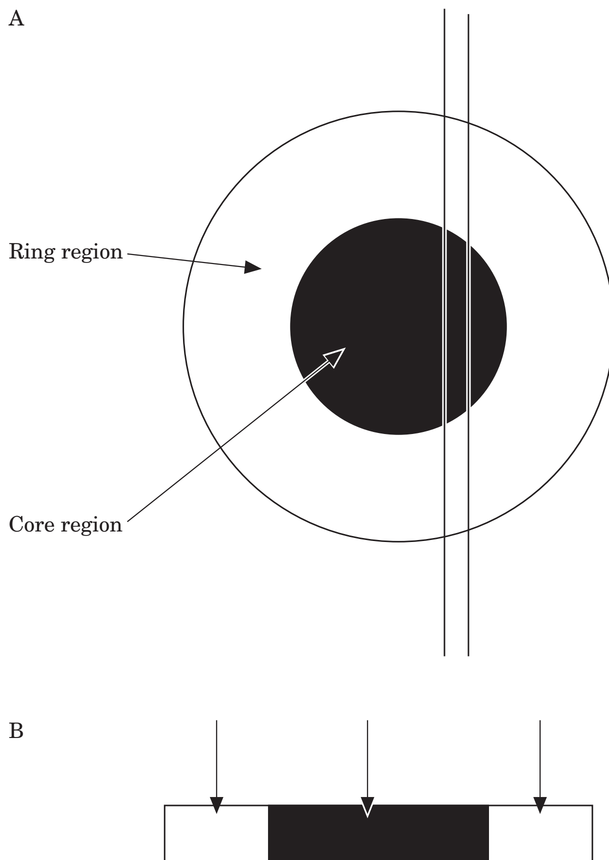


Fig. 1. Diagrammatic representation of the cartilage explant. (a) Top view of cartilage explant: As viewed from above the articular surface, the 4 mm cartilage explant contains a directly impacted region (black area) of 2 mm diameter in the center of the disk, where the indentor damages the cartilage matrix. The surrounding region (white area) is not directly impacted by the mechanical indentor apparatus. In separated samples, the gray inner core is removed from the surrounding ring by a 2 mm biopsy punch. For staining the samples with fluorescent nucleotides for the TUNEL assay, 6 μm sections were taken of the frozen cartilage disk. Sections were taken as two groups of four sections, where groups were taken 1.25 and 1.75 mm from the circumference of the disk as indicated by the two vertical lines. (b) Cross-sectional view of cartilage explant: as seen in cross-section, the 6 mm slices of cartilage used for staining contained both the directly impacted core (black) and the surrounding unimpacted ring (white) regions in unseparated samples. Pictures were taken at the three locations denoted by arrows, giving photos of both sides of the ring, as well as the core region.

Fluoview 2.1, with appropriate filters for PI and fluorescein. Pictures were taken under a 10× objective through a camera linked to the Fluoview system. This program allowed for the same picture to be viewed with both stains merged, eaving a dichromatic field, or separated, allowing for either monochromatic field. Photos were saved to disk, both as merged images and as isolated fluorescein images, allowing for an accurate representation of total vs positively TUNEL stained (TUNEL+) cells. Also, the location within the cartilage sample (core vs ring) was noted for each photo. Each full cartilage section was photographed in three locations (Fig. 1), producing two ring images and one core image.

The quantitation of cells was performed using Scion Image version 1.62 (Scion Co., Frederick, MD, previously named NIH Imaging 1.62). Within each image three random but uniform regions were selected for counting. The uniform areas for counting were 200×200 Scion Image pixel units. Great care was taken to select regions at uniform depth (from 50–75 pixels) below the cartilage surface to avoid non-specific TUNEL positive staining that was present at the surface of many sections including those that had received no load. Since the pictures used for counting total and TUNEL+ cells were simply different treatments of the same image, alignment of the regions for counting in the two images was aided by pixel location markers in Scion Image.

Sections analysed for cell morphology to determine the mode of cell death were analyzed using a Nikon fluorescent microscope under a 100× objective (Eclipse TE 300 inverted microscope, Nikon, Japan). This microscope excites both fluorescent and Hoechst No. 33342 dyes, allowing for qualitative observations of these samples.

ANALYSIS OF NITRIC OXIDE CONCENTRATION

The Griess diazotization reaction was used as a spectrophotometric assay for the determination of nitrite in our media and digested cartilage samples[16]. Nitrite and nitrate are spontaneous by-products of nitric oxide *in vivo*. Our samples (100 μl) were treated with 10 μl nitrate reductase (25 mU/10 μl) and 10 μl NADPH in 10 mM EDTA, and incubated in the dark at room temperature for 30 min to convert nitrate to nitrite. To these solutions were added 70 μl of N-(1-napthyl)ethylendiamine dihydrochloride (1 mg/ml) and 70 μl of sulfanilic acid (10 mg/ml 5% phosphoric acid), as prepared by Molecular Probes. After 20 min of incubation at room temperature in the dark, the samples' optical densities at 548 nm were measured. Standard curves were generated using sodium nitrite either in blank culture media or in a papain solution, according to the given sample's origin.

STATISTICS

Our percentage cell death analysis considered each cartilage disk to be an individual statistical unit. Six counts from each section's two ring photos were pooled and averaged together, as were the three counts from each core region. For each disk, data was usually obtained from four sections cut from each of two different depths. The data were analysed by ANOVA, General Linear Model (Minitab Statistical Software, Minitab, Inc., State College, PA), with respect to duration of impact and time of culture post-impact. For each incubation period, a least squares mean with standard error for each load level was calculated to determine the percentage of cell death. Significance was $P<0.05$.

Results

EFFECTS OF INCREASING IMPACT DURATION

Increased levels of cell death (TUNEL +) with the increase of impact duration were repeatedly seen in all three groups (3, 6 and 21 days) in post-impact culture and were consistent with our previous findings[7]. After 3 days of post-impact, the percentage of cells positive in the TUNEL assay in cores impacted for 0, 20, and 120 min were 2.4, 15.0, and 25.2 respectively (Table I).

Table I
Percentage of cell death in cores and rings of cartilage explants cultured intact after cyclical mechanical impacts

Post-impact culture	Location	No impact	20 min of impact	120 min of impact	*P* values
3 days	Core	2.4±−2.1	15.0±−2.2	25.2±−2.1	<0.001
3 days	Ring	9.1±−2.4	13.3±−2.3	13.2±−2.4	0.380
6 days	Core	7.8±−8.6	35.1±−5.6	45.9±−4.8	0.004
6 days	Ring	5.9±−6.0	27.9±−4.5	27.3±−3.4	0.014
21 days	Core	4.9±−4.2	38.9±−4.2	65.3±−7.3	0.006
21 days	Ring	3.1±−4.4	35.8±4.2	61.3±−7.3	<0.001

Cartilage disks were harvested, subjected to impact loads, cultured intact, and analysed for numbers of cells which gave a positive reaction in the TUNEL assay, as described fully in the Methods section. Significant differences with respect to load were found for cores at all times of post-impact culture and for rings after 6 days and after 21 days post-impact culture. After 21 days, there was no significant difference in percentage of dead cells in the core vs the ring of these explants cultured intact. Analysis of all of the data combined demonstrated significance with respect to both load and days of post-impact culture at $P<0.001$.

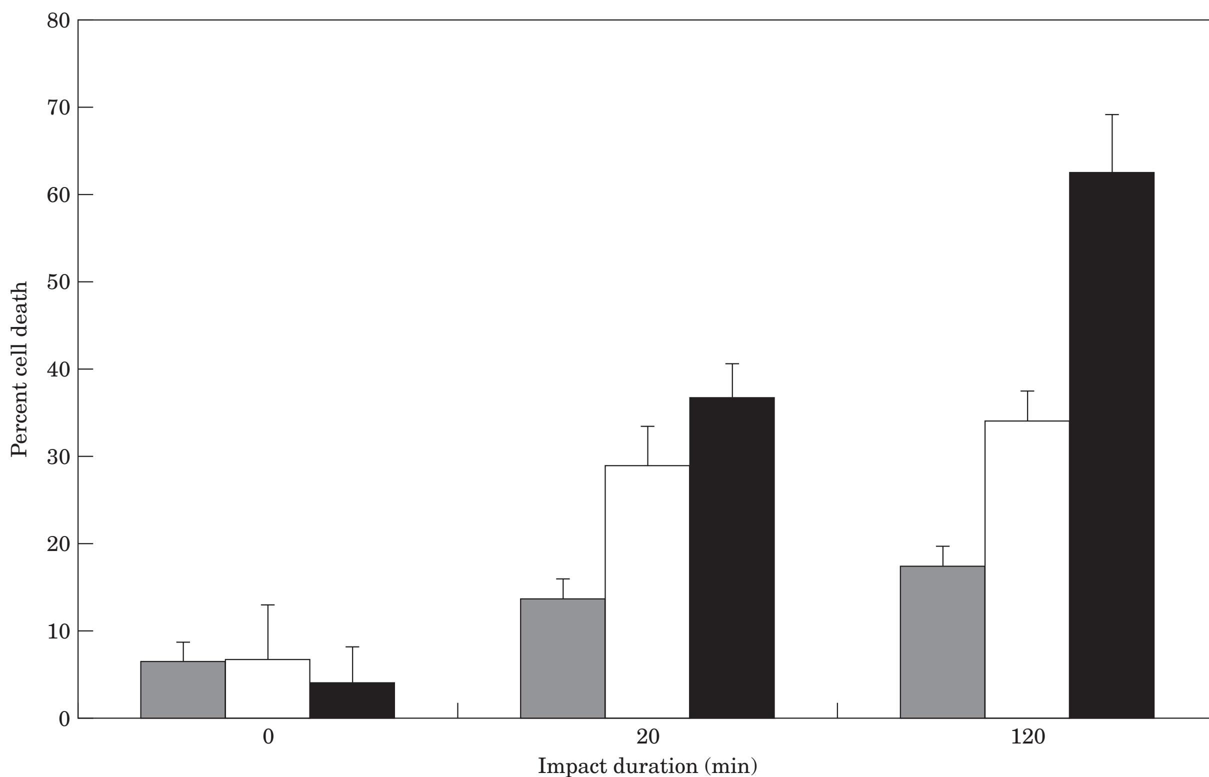


Fig. 2. Percent cell death in response to increased duration of impact. Cartilage disks were harvested, subjected to impact loads, and cultured intact as described in Methods. The percentage of cell death was determined by the TUNEL assay which is fully described in the Methods. The data are presented as mean±standard error of the mean. Total cell death in cartilage explants increased significantly with increasing duration of impact (0, 20, and 120 min) for all periods of incubation post-impact. For damaged explants (20 and 120 min of impact), extent of cell death increased with increasing duration of post-impact culturing (3, 6, and 21 days; ▨, □ and ■, respectively.

EFFECTS OF INCREASING CULTURE TIME POST-IMPACT

In previous experiments in our laboratory, the rate of cell death observed after incubation post impact was followed for only 6 days. In these experiments, the incubation was extended to 21 days after impact. The percentage of dead (TUNEL +) cells was found to be promoted also by the duration of post-impact culture. For explants impacted for 120 minutes, the total number of cells within all areas of the disk which stained positively in the TUNEL assay was 17.3±2.2% after 3 days of culture, and continuously increased to 33.9±3.4% and 62.5±6.6% at 6 and 21 days respectively (Fig. 2).

Furthermore, in explants incubated for 21 days, cell death did not remain localized to the directly impacted areas, as it had in samples incubated for three days. Rather, in two independent experiments with extended incubation periods, non-loaded ring regions within an impacted sample showed cell death at similar levels to those core regions directly impacted. In samples impacted for 120 minutes and cultured for 3 days post-loading, the non-separated ring regions averaged 13.2±2.4%, only about half of the 25.2±2.1% cell death percentage observed in the core regions. However, after 21 days of incubation, the percentage of cell death in these non-impacted perimeter regions was 61.3±7.3%, not

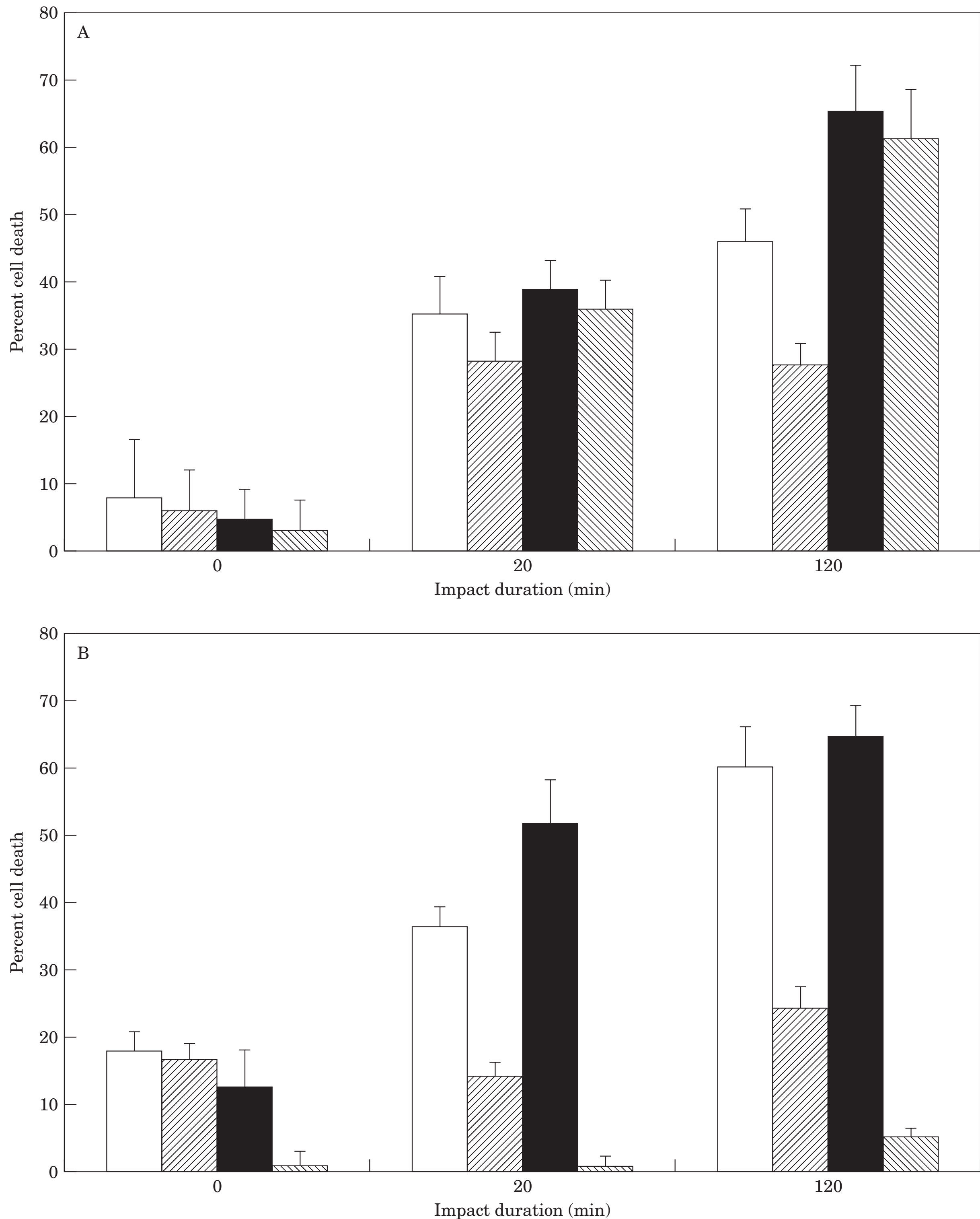


Fig. 3. Cell death in rings surrounding impacted cores and cultured intact or separately post-impact. (A) Percent cell death in rings vs cores in intact samples: cartilage disks were treated and analysed as described for Fig. 2, except that the percent of cell death is shown separately for impacted cores and surrounding rings. The percentage of cell death, as determined by the TUNEL assay in cartilage explants cyclically impacted, was not significantly different between directly impacted (core) and unimpacted (ring) regions after 21 days of post-impact culture, in samples with cores and rings kept intact during culturing. The pattern shown indicates a loss of location-dependent cell death with increased duration of incubation in non-separated cartilage explants. (B) Percent cell death in rings vs cores cultured separately: cartilage disks were treated and analysed as described for Fig. 2, except that immediately after impact and prior to culture, impacted cores and the surrounding rings were separated and placed into different culture dishes. The percentage of cell death, as determined by the TUNEL assay in cartilage explants cyclically impacted, was significantly greater in directly impacted (core) regions than the surrounding unimpacted (ring) regions. Unloaded control samples showed no significant difference between cores and rings, with the exception of reduced cell death in 21 day rings. Cores: 6 days, □; 21 days, ■. Rings: 6 days, ▨; 21 days, ▧.

statistically different from the impacted core region's 65.3±6.9% [Figs 3(a), 4(a), (b), Table I].

LOCALIZED CELL DEATH AND APOPTOTIC CELLS

In cartilage disks impacted for 120 minutes and incubated for 21 days, chondrocytes staining positive in the TUNEL assay could be seen throughout the depth of the disk in both the surrounding ring and core [Fig. 4(a), (b)]. Using Hoechst dye, both apoptosis-like and necrosis-like nuclear morphology were observed in impacted disks. At day 3, the core region had an estimate of 5 to 10% of the cells exhibiting fragmented and shrunken nucleus. After 21 days, however, the rings of samples had about 35% of cells showing clear nuclear morphology consistent with apoptosis.

EFFECTS OF SEPARATING CORE AND RING REGIONS

For samples whose core regions were removed from the surrounding ring directly following impact, the core regions produced the expected pattern of increased cell death with increased impact duration and increased time of culture post-impact. Observations were made at 6 days post-impact and at 21 days post-impact and cell death in cores loaded for 120 min reached 60.1±6.0% and 64.8±4.4%, respectively. The percentage of cell death in the ring regions of these separated samples, in contrast, remained at the control levels in samples loaded for 20 min and increased only slightly in samples loaded for 120 min [Figs 3(b), 4(c), (d)].

EFFECTS OF CO-INCUBATING IMPACTED CORES WITH NON-LOADED DISKS

In non-loaded samples that were cultured together with 120-min impacted cores for 21 days, the percentage of dead cells (2.5%±0.4%) showed no increase over that of unimpacted control samples incubated alone for the same duration (4.1%±0.7%). The core regions were qualitatively analysed as having undergone considerable cell death.

NITRIC OXIDE CONCENTRATION

No significant differences were found between the concentrations of nitric oxide in loaded vs non-loaded samples. In papain digests of non-loaded cartilage disks, we found a nitric oxide concentration of 0.25±0.14 μmol/mg wet cartilage after 21 days, as compared to the 0.20±0.12 μmol/mg concentration of samples loaded for 120 min. A similar trend was found in the culture media collected during the 21 days of incubation. Media from the non-loaded sample yielded nitric oxide at 0.009±0.005 μmol/mg, whereas those loaded for 120 min contained 0.008±0.004 μmol/mg.

GLYCOSAMINOGLYCAN CONCENTRATION

Glycosaminoglycan concentrations observed in this experiment ranged from a low of 19.5±2.3 μg/mg wet cartilage to 41.5 μg/mg wet cartilage but were not statistically different with respect to load or time of culture.

TOTAL CELL NUMBERS

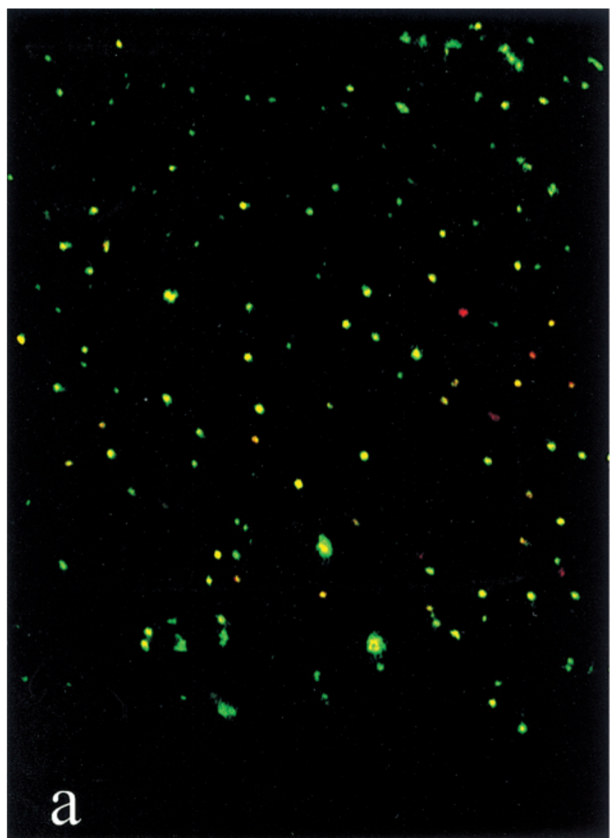
Within each group, the total number of chondrocytes counted in all sections was totaled and divided by the total number of frames counted. Total cell numbers varied from 18.4 to 36.2 cells/frame in cartilage disks that had received no load and from 17.8 to 35.1 cells/frame in disks that had been loaded for 120 min.

Discussion

In this study, we investigated the time-dependent and location-dependent responses of cartilage explants to damage caused by the application of impact loads. The results were consistent with our previous study that demonstrated increased cell death with increasing severity of impact loading within the loaded core region[7], and with the study of Torzilli *et al.*, who have also demonstrated that mechanical damage *in vitro* leads to cell death[17]. In this study, we expanded the post-impact culture time to up to 21 days. The important findings are that cell death increased with increased post-impact culture time, and that cell death spread to non-impacted regions. Prolonged culture alone was not responsible for the loss of cell viability. Instead, we can support the hypothesis that the increase in cell death over time was related to impact and that the cause of cell death continued to operate long after the initial damage from impact loading had occurred.

The location-dependent responses in cell death were observed in comparing explants cultured for 3 days against those cultured for longer duration. After 3 days, even the most heavily damaged matrices (120 min of loading) showed the majority of TUNEL+ cells in the directly impacted core region, and most of the death was also confined to the surfaces of the explant. However, after 6 days and 21 days of incubation, the levels of TUNEL+ cells increased in the surrounding ring regions. In fact, by 21 days the surrounding regions showed the same extent of cell death as the directly impacted core. Furthermore, the propagation of cell death through the depth of the sample increased as well. This observation of the locational spreading of TUNEL+ cells indicated that a diffusible signal might be present in the damaged cartilage, allowing for the eventual spread to the outer regions. To test this idea, cores were removed from the surrounding ring immediately after termination of impact and cultured separately under identical conditions. When this was done, there was a marked decrease in cell death in the ring regions as compared with the ring regions of non-separated samples. Separated rings, on average, remained at approximately control levels, even after 21 days of culture, while the core regions, in addition to showing the expected increases in cell death with increased impact and culture times, also displayed the expected propagation through the depth of cartilage with increased culture time. It was concluded that propagation of a diffusible signal from core to ring continues after the time of impact and that diffusion of this signal was prevented by physical separation of ring from core.

In our earlier study of the nature of cell death in cartilage explants subsequent to impact damage[7], it was shown that chondrocytes with characteristics of cell death by necrosis could be detected within 2 h after impact. TUNEL positive cells were not present in these explants after 4 h but were detected after two days of culture. At that time, both necrotic and apoptotic cells were positive in the TUNEL assay indicating that the TUNEL assay was not specific for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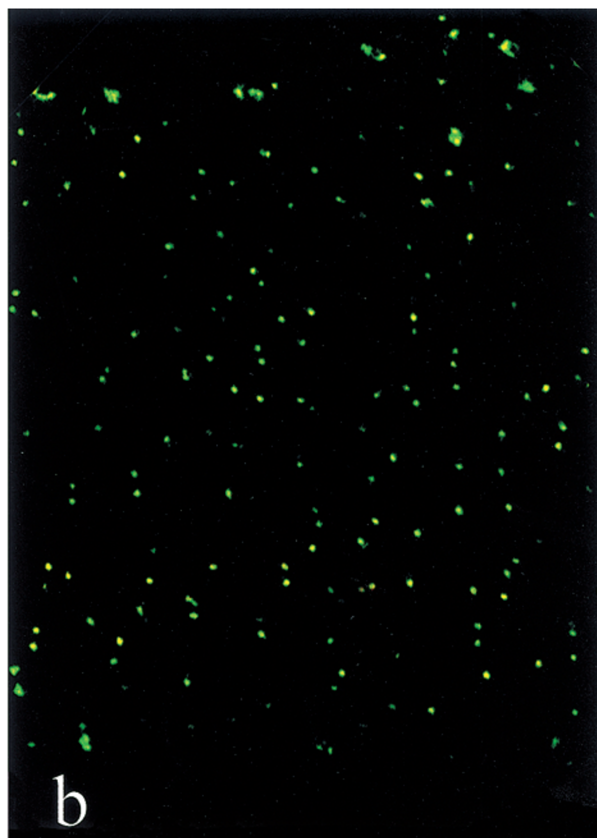
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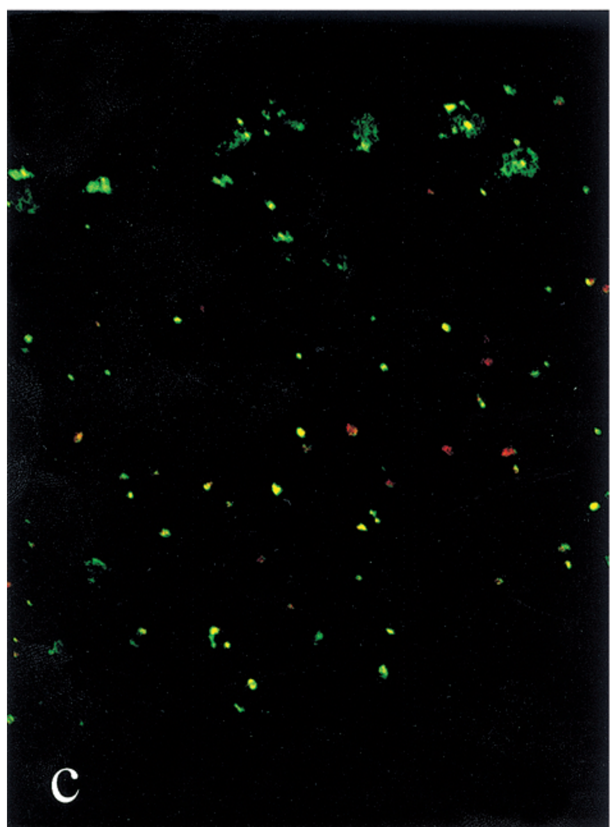
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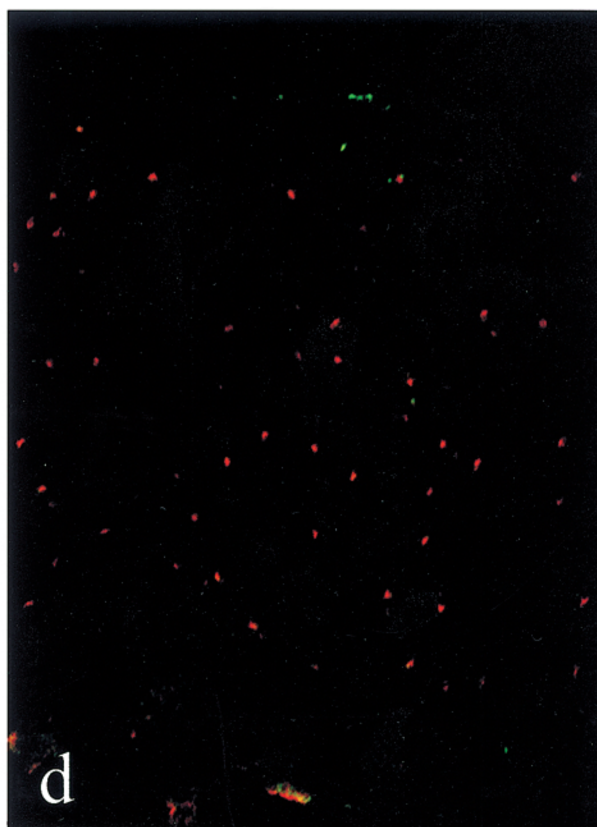
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apoptosis. Others have also observed that TUNEL staining does not distinguish apoptosis from necrosis[18]. Thus, in this study, a positive reaction in the TUNEL assay was taken to mean cell death without further distinction as to the mechanism of that cell death. Therefore, questions regarding the mode of cell death, necrosis or apoptosis, as cell death increases with time, remain. However, a semi-quantitative evaluation of nuclear morphology using Hoechst dye was consistent with an increase in true apoptotic cells over time. Apoptosis should be rapid. Studies have shown that apoptosis in chondrocytes may occur within 6 h[12], or even less time[19]. Thus a delayed appearance of apoptosis is consistent with the continued signaling of cell death long after the initial insult. The apparent accumulation of TUNEL positive cells over time may reflect the absence of phagocytic cells in the cartilage that permits dead cells to persist in the cartilage matrix, as suggested by Lotz *et al.*[20]

Further studies will be directed toward elucidating the nature of this soluble factor, however previous reports have given reason to consider NO[12] or a cytokine, such as IL-1β[21], TNF-α[21,22], or Fas ligand[14]. Studies have indicated that nitric oxide concentrations in synovial fluid and cartilage increase during OA[23,24]. NO has been implicated in the development of the joint pathology associated with NO[25,26]. Donors of NO induced cell death in *in vitro* studies of chondrocytes, but the relationship of NO to cell death in chondrocytes is complex and not entirely clear (reviewed by Lotz *et al.*[20]). Furthermore, high levels of IL-1β[21], Fas ligand[14] and increases in levels of TNF-p55, the 55 kD analog of TNF-α[13] have been found in osteoarthritic cartilage and synovial fluid. Studies have also indicated that synovial fibroblasts in OA patients express the receptor to TNF-p55 at much higher rates than normal fibroblasts, and suggest that perhaps the same may be true for OA chondrocytes[27]. These cytokines have already been shown to suppress the synthesis of Type II collagen and proteoglycan core protein, key steps in the degeneration of cartilage matrix during OA[27]. The ability of NO and each of these cytokines to initiate the caspase cascades necessary for apoptosis in mammalian cells gave rise to each being a suspect as the death signal indicated above. Preliminary results did not implicate NO but additional experiments to determine if inhibitors of nitric oxide synthetases will prevent the spread of cell death from the site of initial impact will be required before a role for nitric oxide can be rejected.

Furthermore, co-culture of non-impacted explants with cores of impacted disks for 21 days failed to induce cell death in the explants. Since the cores exhibited cell death as expected, this may indicate that the signal was too diluted in the media to have any effect, or that something in the media, not present in the matrix, was reacting with the soluble signal to inactivate it. Another possibility is that the signaling molecule was retained inside the cartilage matrix. Walker *et al.*[19], who observed a limited amount of spreading of cell death from the site of injury in wounded embryonic chick sterna, considered the possibility that the loss of a survival signal, and/or progressive damage to the cartilage matrix, was responsible.

Proteoglycan concentration in osteoarthritic samples early in the disease may show little difference from normal tissue, although loss of proteoglycan is a defining characteristic of more advanced lesions. Cyclically impacted cartilage may show a time-dependent and damage intensity-dependent response for GAG synthesis with stimulation of synthesis at early times and milder loading protocols but a decrease in glycosaminoglycan synthesis in cartilage explants with extensive cell death[2,7]. Likewise, both apoptotic chondrocytes and a decrease in chondrocyte numbers has been reported for osteoarthritic cartilage. Thus it is somewhat surprising that in this study, no significant decreases in glycosaminoglycan content or in numbers of cells was observed. However, large variation among cartilage samples may have obscured small differences due to impact damage. Furthermore, proteoglycan turnover and persistence of apoptotic cells within cartilage matrix are also factors to consider

When extrapolating from these *in vitro* results to the *in vivo* situation several things should be kept in mind. *In vivo*, in normally congruent articular joints, deformation and local strain within the cartilage may be limited while *in vitro*, there are likely to be large stress amplitudes and gradients in the vicinity of the indenter. Furthermore, *in vivo*, cartilage is attached to the subchondral bone while in these experiments, cartilage disks were excised from the bone. The strain and stress distribution of a loaded cartilage disk would be somewhat different from the osteochondral plug, especially in the deep zone. The removal of subchondral bone would, however, not much affect the strain field near the articular/contact surface in which the dead cells first appear.

Finally, studies in humans suggest that the contact stress in joints under normal exercises is from 0 to 5 MPa at a stress rate of 20 MPa/s, and may reach 20 MPa during jumping, lifting, and other intensive exercises[28,29]. Studies in the pig[30] suggested that the contact stress in the quadrupeds was 0–2 MPa lower than in humans. Due to the variety of gait patterns and anatomical differences between animals, the parameters used in this study may be comparable to that seen *in vivo* and are within the tolerance of a normal joint but might challenge repair or otherwise compromised cartilage. The cyclic impacts were employed in this study, however, to mimic the abnormal loads in an unstable knee with ACL deficiency or a subluxed hip with dysplasia. The results of this study can shed some lights on cell death and damage due to the introduction of joint abnormalities which have been shown in several studies to trigger OA.

Fig. 4. Photographs of stained sections of core and ring regions cultured contiguously or cultured separately. Photographs were obtained as described in Methods from selected specimens representing the data shown in Fig. 3(a) and (b). Dead cells stained positive in the TUNEL assay and are stained green in the photographs. Counterstaining of all cells was with propidium iodide and is detected as red fluorescence in cell that were negative in the TUNEL assay. (a) The core region of a cartilage sample impacted for 120 min, after 21 days of culture. Dead cells, shown staining green as a result of a positive reaction in the TUNEL assay, are seen throughout the depth of the disk instead of the localized regions seen in a 3-day impacted sample. (b) The ring region of a cartilage sample impacted for 120 min, after 21 days of culture. The extent of cell death most closely resembles the core of the same sample (a). (c) The core region from a cartilage explant whose core was removed from the surrounding ring region immediately after 120 min of impact damage and cultured separately for 21 days. Cell death is seen throughout the depth of the cartilage section. (d) The ring region from a cartilage explant in which the ring was removed from the core it surrounded immediately after 120 min of impact and cultured separately for 21 days. The extent of cell death is much less than in the core region of the same sample (c).

Considering the correlation between matrix damage and the onset of OA, this study may help us understand the factors causing OA in cartilage. A loss of chondrocyte viability may play a significant role in the progression of OA, and the possible finding that intercellular signaling is involved in the early onset of osteoarthritis can provide future researchers with a potential way of intervening in the transmission of the signal, and thus of controlling the expression of OA. However, due to the *in vitro* nature of our study, it is difficult to draw a direct connection between our findings and OA at this time. Further studies into the nature of the signaling mechanism in *in vitro* samples, as well as analysis as to the presence and function of this intercellular signal in osteoarthritic samples *in vivo*, will be important factors in elucidating the *in vivo* spread of the disease.

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