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ARTICLES

Hydrostatic Pressure Induces Apoptosis in Human Chondrocytes From Osteoarthritic Cartilage Through Up-Regulation of Tumor Necrosis Factor-α, Inducible Nitric Oxide Synthase, p53, c-myc, and bax-α, and Suppression of bcl-2

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Abstract Hydrostatic pressure (HP) is thought to increase within cartilage extracellular matrix as a consequence of fluid flow inhibition. The biosynthetic response of human articular chondrocytes to HP in vitro varies with the load magnitude, load frequency, as well as duration of loading. We found that continuous cyclic HP (5 MegaPascals (MPa) for 4 h; 1 Hz frequency) induced apoptosis in human chondrocytes derived from osteoarthritic cartilage in vitro as evidenced by reduced chondrocyte viability which was independent of initial cell densities ranging from 8.1×10^4 to 1.3×10^6 cells ml^{-1}. HP resulted in internucleosomal DNA fragmentation, activation of caspase-3, and cleavage of poly-ADP-ribose polymerase (PARP). At the molecular level, induction of apoptosis by HP was characterized by up-regulation of p53, c-myc, and bax-α after 4 h with concomitant down-regulation of bcl-2 after 2 h at 5 MPa as measured by RT-PCR. In contrast, β-actin expression was unchanged. Real-time quantitative RT-PCR confirmed a HP-induced (5 MPa) 1.3–2.6 log-fold decrease in bcl-2 mRNA copy number after 2 and 4 h, respectively, and a significant increase (1.9–2.5 log-fold) in tumor necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS) mRNA copy number after 2 and 4 h, respectively. The up-regulation of p53 and c-myc, and the down-regulation of bcl-2 caused by HP were confirmed at the protein level by Western blotting. These results indicated that HP is a strong inducer of apoptosis in osteoarthritic human chondrocytes in vitro. J. Cell. Biochem. 87: 266–278, 2002.

Key words: gene expression; hydrostatic pressure; mechanotransduction; programmed cell death; signaling pathways

Osteoarthritis (OA) is a progressive and debilitating condition of synovial joints, which is clinically significant because of its high morbidity resulting from destruction of articular cartilage and bone. Cartilage aging and OA appear to be two distinct processes. However, pathologic processes consistent with OA are found almost universally among the elderly [Malemud, 1999]. While OA pathogenesis is believed to arise by multifactorial changes in cartilage and bone, the initiating factors responsible for the earliest OA lesions of articular cartilage remain unknown [Malemud and Goldberg, 1999]. One finding common to both OA and

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aged cartilage is reduced cellular content [Hashimoto et al., 1997; Adams and Horton, 1998]. Whether the earliest pathological changes in OA cartilage involves loss of chondrocyte cellularity and viability by programmed cell death (apoptosis) is debatable [Hashimoto et al., 1998; Aigner et al., 2001; Héraud et al., 2000; Kim et al., 2000; Kouri et al., 2000; C-T. Chen et al., 2001; D'Lima et al., 2001].

Chondrocyte apoptosis has been linked to structural changes in mitochondrial membranes and low levels of *bcl-2* [Feng et al., 1998], typical of apoptosis in other tissues [Adams and Cory, 1998; Kobayashi et al., 1999]. Bcl-2 protein may function by inhibiting the release of cytochrome C from mitochondria and/or by inhibiting the activity of caspases [Green and Reed, 1998; O'Connor and Strasser, 1999]. The induction of apoptosis in cultured articular human chondrocytes derived from OA cartilage has been largely unexplored although previous studies have indicated the profound influence of nitric oxide (NO) and the interaction of NO with oxygen radicals in regulating apoptosis in chondrocytes [Blanco et al., 1995; Notoya et al., 2000] as well as the potential role of tumor necrosis factor-α (TNF-α) as initiating apoptosis in chondrocytes [Aizawa et al., 2001].

It is well known that two environmental factors influencing skeletal tissue development and differentiation are tissue oxygen tension and mechanical loading [Bourret and Rodan, 1976]. Articular cartilage from normal weight-bearing joints is subject to complex load waveforms with hydrostatic pressure (HP) magnitudes as high as 10–20 MegaPascals (MPa) [Veldhuijzen et al., 1979]. The applied load signal can be broken down into several components. These include, load magnitude, frequency, and duration. HP is thought to build up within cartilage extracellular matrix as a result of fluid flow inhibition [Lippiello et al., 1985; Thonar and Kuettner, 1987]. Frequencies in the range of 10^{-4}–10^{-3} Hz are associated with significant fluid flow. By contrast, frequencies ranging from 0.01–1.0 Hz were associated with reduced fluid flow and increased HP. These frequencies are consistent with those generated during normal gait.

The biosynthetic response of articular chondrocytes to changes in HP has been shown to vary with the parameters of loading. Intermittent pressures appear to favor stimulation of chondrocyte metabolism more than does continuous pressure [Klein-Nulend et al., 1986]. For intermittent pressures, the cellular responses in vitro appear to be frequency-dependent [Sah et al., 1989; Parkkinen et al., 1993a,b; Lammi et al., 1994; Steinmeyer and Knue, 1997]. Frequencies of 0.5 and 0.25 Hz are associated with augmented proteoglycan synthesis whereas lower frequencies (1.7×10^{-2} Hz) inhibited proteoglycan synthesis [Lammi et al., 1994]. Cyclic compression of articular cartilage at 0.1 Hz and higher was consistently associated with an increase in cartilage oligomeric matrix protein (COMP) as well as fibronectin synthesis [Wong et al., 1999].

The chondrocyte response also depends on the magnitude of the HP employed. In this regard, HP in the range of 5–10 MPa are associated with increased proteoglycan synthesis when compared to pressures less than 1 MPa or greater than 15 MPa. HP ranging from 0.1 to 5 MPa appear to be useful in these experimental systems and these pressures include stress values of 0.8–6.3 MPa, which have been measured in the knee joint during normal walking [Maquet et al., 1975; Mathews et al., 1977].

Mechanical overloading of cartilage has been implicated in the pathogenesis and progression of OA [Quinn et al., 2001]. However, the earliest pathological OA cartilage changes are likely to result from continuous loading of joint cartilage as the patterns of osteofemoral cartilage degeneration are often superimposable when multiple specimens are compared [Sachs et al., 1982]. Thus, if apoptosis is a candidate target for chondrocyte dysfunction then HP loading of chondrocytes derived from low or non-weight bearing regions of the osteofemoral head with stress values measured in the normal joint during normal walking (see above) would be hypothesized to induce apoptosis in these chondrocytes.

In the present study, we examined whether HP could induce apoptosis in human articular chondrocytes derived from OA cartilage. Initial experiments focused on what frequencies and time periods for pressure-loading [C-T. Chen et al., 2001] would be sufficient to induce apoptosis. Our results showed that HP induction of apoptosis in vitro was load- and time-dependent and characterized by loss of chondrocyte viability, internucleosomal DNA fragmentation, activation of procaspase-3, and cleavage of poly-ADP-ribose polymerase (PARP). RT-PCR

showed that apoptosis in human chondrocytes induced by HP was accompanied by up-regulation of *p53*, *c-myc*, and *bax-α* gene expression. Increased p53 and c-myc protein synthesis was also detected by Western blotting. In contrast to these results, HP downregulated *bcl-2* gene expression and bcl-2 protein synthesis. Downregulation of *bcl-2* was confirmed by quantitative real-time RT-PCR. Further, quantitative real-time RT-PCR showed that HP induction of apoptosis in human chondrocytes resulted in up-regulation of *TNF-α* and inducible nitric oxide synthase (*iNOS*) gene expression.

MATERIALS AND METHODS

Cartilage

Human articular cartilage was obtained from adults undergoing a variety of orthopaedic surgical procedures, including total joint arthroplasty at University Hospitals of Cleveland. The "resident" cartilage was harvested from osteoarticular femoral heads. This cartilage is not morphologically normal but is topographically isolated from the principal weight-bearing surface of the femoral head and consists mainly of discolored and partially fibrillated cartilage [Sachs et al., 1982]. The cartilage was removed by slicing thin layers with a scalpel until the underlying subchondral bone was exposed. Osteochondrophyte spurs were scrupulously avoided. The cartilage slices were placed in PBS containing 1% penicillin–streptomycin on ice prior to chondrocyte isolation.

Enzymatic Digestion of Cartilage and Chondrocyte Cultures

Cartilage slices were digested in 0.15% (w/v) pronase for 1 h, followed by 0.15% (w/v) *Clostridium histolyticum* collagenase overnight at 37°C. The isolated chondrocytes were maintained in nutrient F-12 media (Gibco, Grand Island, NY) containing L-glutamine, supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and fungizone. In a typical experiment, chondrocytes were plated at an initial density of 10^6 cells per 100 mm tissue culture dish which were allowed to reach ~90% confluency.

HP Loading of Chondrocytes

Approximately 2×10^6 chondrocytes in culture medium (see above) were placed in sterile heat-sealable bags (Kapak Corporation, Minneapolis, MN) and subjected to HP employing a servopneumatic materials testing system (SmartTestII, Enduratec Systems Corp., Eden Prairie, MN) which was fully computer-controlled (QuikTest Software, Enduratec Systems Corp.). In this test system, the axial force applied by the pneumatic actuator is directly proportional to the HP within the fluid-filled chamber, which was validated using pressure-sensitive film (Fuji Film, East Hanover, NJ). This device is capable of applying HP of up to 1,600 psi (≅11 MPa) to cell cultures using either continuous or intermittent (cyclic) waveforms (Angele et al., 2002] and is maintained in a large capacity CO_2 incubator (Hotpack Corp., Philadelphia, PA). Chondrocytes were either maintained in the sealed bags for 0–4 h (i.e., non-loaded chondrocytes) or subjected to HP employing a 1 Hz continuous sinusoidal waveform at 2.5 or 5 MPa for up to 4 h.

Isolation of Total RNA/Messenger RNA (mRNA) and Reverse Transcription (RT)

Total RNA or mRNA were isolated from either non-loaded or pressure-loaded chondrocytes. The isolation protocol followed specifications as supplied by the manufacturer (Qiagen, Valencia, CA). Total RNA or mRNA was reverse-transcribed using SuperScript II reverse transcriptase (Life Technologies, Gibco BRL, Gaithersburg, MD). The primers (Stratagene, La Jolla, CA) and RT-PCR conditions followed protocols supplied by the manufacturer (Life Technologies Gibco BRL).

Quantitative Real-Time RT-PCR

We employed quantitative real-time RT-PCR with internal fluorescent hybridization probes in the ABI Prism 7700 Detection System (ABI, Foster City, CA) to quantify relevant apoptosis gene transcription. This technique affords a sensitive and specific quantification of individual RNA transcripts [Hartel et al., 1999]. An RT-PCR assay for human ribosomal 18S (R18) was employed in order to normalize gene expression. TaqMan™ PCR primers (ABI/Perkin Elmer [PE] Biosystems, Foster City, CA) and probes for R18 genes were designed using Primer Express™ (ABI/Perkin Elmer [PE] Biosystems). A target-specific RT primer was also designed for each gene. The primer and probe sequences for R18, iNOS, bcl-2, and TNF-α are shown in Table I.

TABLE I. Primer and Probe Constructs for Quantitative Real-Time RT-PCR

R18
R18 RT primer: GACGGTATCTGATC
R18-1014 (reverse primer) G: 5′-CAT TCT TGG CAA ATG CTT TC-3′
R18-948 (forward primer): 5′-CGC CGC TAG AGG TGA AAT TC-3′
R18 Probe (972T): 5′-6FAM-ACC GGC GCA AGA CGG ACC AGA-TAMRA-3′
iNOS
iNOS RT-primer: 5′-CTCTGGTCAAAC-3′
iNOS (forward primer) 5′-AGCGGGATGACTTTCCAAGA-3′
iNOS (reverse primer) 5′-ATAATGGACCCCAGGCAAGATT-3′
iNOS probe: 5′-6FAM-CCATAAGGCCACCGGGATTTTAACTTGCAG-TAMRA-3′
bcl-2
bcl-2 RT primer: 5′-CTGCACATTTATTG-3′
bcl-2 (forward primer): 5′-AGAACCTTGTGTGACAAATGAGAAC-3′
bcl-2 (reverse primer): 5′-TACCCATTAGACATATCCAGCTTGA-3′
bcl-2 probe: 5′-6FAM-AGACATCAGCATGGCTCAAAGTGCAGCT-TAMRA-3′
TNF-α
TNF-α RT primer: 5′-GGTTTGCTACAACA-3′
TNF-α (forward primer): 5′-AGGCGGTGCTTGTTCCTCA-3′
TNF-α (reverse primer): 5′-GTTCGAGAAGATGATCTGACTGCC-3′
TNF-α probe: 5′-6FAM-CCAGAGGGAAGAGTTCCCCAGGGAC-TAMRA-3′

All probes were labeled with 5-carbofluorescein (FAM) at the 5′ end and *N*′,*N*′,*N*′*N*′-tetramethy-6-carborhodamine (TAMRA) at the 3′ end (PE). The proximity of the dye (FAM) and the quencher (TAMRA) on the intact probe prevents detection of any fluorescence. During the course of PCR, degradation of probe takes place, which in turn allows the release and detection of FAM [Holland et al., 1991]. The PCR reaction for all amplifications was similar and carried out as described by the procedure supplied by PE Biosystems (Perkin Elmer). Conditions for PCR were similar for all products (1 cycle, 50°C × 2 min and 1 cycle, 95°C × 10 min, 40 cycles of 95°C × 15 s and 60°C × 1 min). The threshold cycle (C_T) for each sample was compared with C_T values of known amounts of a standard DNA constructed for each target and amplified simultaneously. In each sample, *bcl-2*, *TNF-α*, and *iNOS* gene expression was normalized to human ribosomal 18S by employing the $2^{-(\Delta\Delta C_T)}$ conversion. The results were expressed as copies of *bcl-2*, *TNF-α*, or *iNOS* in 10^{10} copies of R18 (equivalent to 10^6 cells).

In some experiments, a duplicate sample tube without enzyme was included as control in order to ascertain the level of DNA contamination in the RNA samples. DNA contamination was found to be negligible.

Chondrocyte Lysate Protein

When the cells reached ~90% confluency, chondrocytes were harvested, resuspended in 300–400 μl cell lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM $NaHPO_4$, 130 mM NaCl, 1% Triton-X 100, 10 mM NaPPi) containing a protease inhibitor cocktail tablet (Boehringer-Mannheim, Indianapolis, IN) and incubated for 30 min on ice. Total cell lysate protein was collected by centrifugation at 10,000*g* and protein concentration determined by a dye-binding assay [Bradford, 1976].

Western Blot Analysis

Western blotting was employed in order to determine whether proteins of relevant apoptosis genes were altered by HP. Chondrocyte lysate protein was treated with protein G-agarose slurry prior to Western blotting. One hundred microliter of protein G-agarose slurry (Gibco) was added to chondrocyte lysate and the sample shaken overnight at 4°C. The agarose beads were centrifuged and transferred to a 12 ml conical tube and washed four times with ice-cold phosphate buffered saline. In all cases, the reagents, kits and protocols for detection of antigen/antibody complexes following SDS–PAGE employed the chemiluminescence ECL+Plus Western blotting detection system (Amersham, Arlington Hts., IL) as previously described [Islam et al., 2001; Singh et al., 2002]. After developing the blots with the ECL+Plus detection system, the chemiluminescent-labeled bands were visualized on Kodak Bio-Max Light Film. The following antibodies were employed: anti-PARP (anti-human-PARP, clone 7D3-6; PharMingen International, San Diego, CA); anti-caspase-3 (anti-CPP32, clone 19; Transduction Laboratories, Lexington, KY); anti-p53 ([pan (polyclonal BMG-1B1)]; Boehringer Mannheim, Indianapolis, IN); anti-c-myc (mouse anti-human c-myc, clone 9E10); Boehringer Mannheim); anti-β-actin (C-2; Santa Cruz Biotechnology, Santa Cruz, CA); anti-bcl-2

(mouse anti-human bcl-2, Ab-1; Oncogene Research Products, Cambridge, MA).

DNA Fragmentation Assay

Internucleosomal DNA fragmentation was measured using the apoptotic DNA ladder assay kit and protocol supplied by Boehringer Mannheim. DNA was electrophoresed on 1.2% agarose gels, which was stained with ethidium bromide. The bands were visualized using a Vilber-Laurmat UV illuminator [Islam et al., 2000].

MTT Assay and Cell Death ELISA

Chondrocyte viability was determined by the MTT-based cell proliferation and viability assay system according to the protocols supplied by the manufacturer (R&D Systems) as previously described [Islam et al., 2000; Singh et al., 2002]. The results of the MTT assay were confirmed by employing the Cell Death ELISA (Boehringer Mannheim). The Cell Death ELISA is a monoclonal antibody based method and is specific for oligonucleosomes produced via the degradation of DNA. Cell death was determined according to the protocol provided by the manufacturer.

RESULTS

Effect of HP on Chondrocyte Viability

Human OA chondrocytes were cultured for 6 h in low-FBS ($< 10\%$ v/v) at various starting cell densities (range, 2.5×10^3–1.3×10^6]. After washing with buffered saline, chondrocytes were maintained in complete medium containing 10% FBS for 24 h. Chondrocyte viability was assessed using the MTT-based cell proliferation and viability assay (Fig. 1). The results in Table II showed that human OA chondrocytes exhibited reduced viability as function of HP and time. The results of the MTT-based cell proliferation assay were confirmed by the Cell Death ELISA (data not shown).

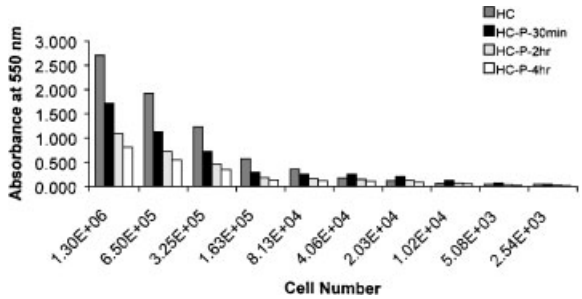

Fig. 1. Hydrostatic pressure (HP) reduces the viability of human OA chondrocytes. Chondrocytes were initiated at the densities shown and viability measured by the MTT assay. HC: non-loaded chondrocytes; HC-P-30 min: 5 MPa, 30 min; HC-P-2: 5 MPa, 2 h; HC-P-4 h: 5 MPa, 4 h. X axis, cell number where 1.30E+06 = 1.3×10^6 cells, etc. Y axis, absorbance at 550 nm. In this representative experiment, the viability of a single culture at each initial density under the conditions and time periods shown was measured.

HP Results in Internucleosomal DNA Fragmentation and PARP Degradation

HP (5 MPa) caused internucleosomal DNA fragmentation as seen on agarose gels (Fig. 2). DNA fragmentation was not detected after 30 min of pressure-loading (Fig. 1, lane 2) in that the pattern of DNA migration was identical to non-loaded chondrocytes (Fig. 2, lane 1). In contrast, extensive DNA fragmentation was seen after 2 h (Fig. 2, lane 3) and 4 h (Fig. 2, lane 4). The pattern of DNA fragmentation was consistent with the apoptosis DNA ladder (Fig. 2, lane M).

PARP degradation was not detected in non-loaded chondrocytes as shown by Western blotting with anti-PARP antibody nor was PARP degradation evident after HP for 2 h at 5 MPa (Fig. 3). PARP degradation was seen after HP for 4 h at 5 MPa (Fig. 3, lane 3). Intact PARP was

TABLE II. Hydrostatic Pressure (HP) Reduced Human OA Chondrocyte Viability

Culture condition	Initial starting cell density ($\times 10^5$) ($\Delta\%$)					
	13	6.5	3.15	1.63	0.813	0.406
HC-P-30 min	−36.4	−40.0	−44.0	−54.5	−37.5	+25.0
HC-P-2 h	−61.8	−70.0	−64.0	−63.6	−50.0	0
HC-P-4 h	−70.9	−75.5	−80.0	−81.8	−75.0	−15.0

Human OA chondrocytes were initiated at varying densities and subjected to HP (5 MPa) for 30 min (HC-P-30 min), 2 h (HC-P-2 h), or 4 h (HC-P-4 h). Chondrocyte viability was assessed by the MTT assay (Fig. 1) and the change in cell viability ($\Delta\%$) of chondrocytes subjected to HP compared to non-loaded chondrocytes cultured for 30 min, 2 h and 4 h in the loading device.

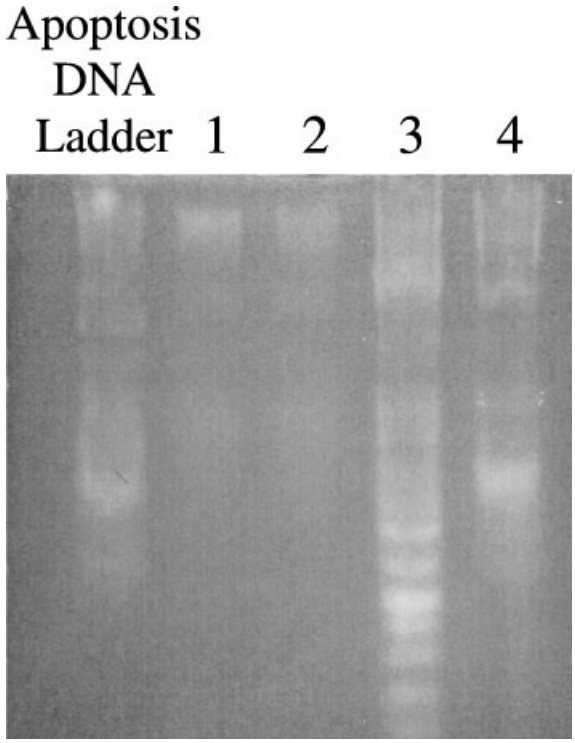

Fig. 2. HP results in internucleosomal DNA fragmentation. Chondrocytes were subjected to HP (5 MPa) or maintained in the loading device (non-loaded chondrocytes) for up to 4 h. Internucleosomal DNA fragmentation was assessed by comparing the pattern of chondrocyte DNA fragmentation to an ''apoptosis DNA ladder'' standard. DNA was electrophoresed on 1.2% agarose gels, stained with ethidium bromide, and the bands visualized using a Vilber-Laurmat UV illuminator. **Lane 1**: non-loaded chondrocytes; **lane 2**, HP, 5 MPa, 30 min; **lane 3**, HP, 5 MPa, 2 h; **lane 4**, HP, 5 MPa, 4 h.

cleaved from the immunoreactive 116 kDa molecule into an immunoreactive 89 kDa PARP fragment (Fig. 3, lane 3). Intact PARP was also detected after HP for 4 h (Fig. 3, lane 3).

The caspase family of cysteine proteases plays a key role in cell proliferation and apoptosis [Elkon, 1999]. Caspase-3 is activated early in apoptosis and appears to be involved in the proteolysis of several important molecules, such as PARP, the sterol regulatory element binding proteins, and bcl-2 [Thornberry and Lazebnik, 1998]. The possible role of caspase-3 in HP-induced apoptosis in human chondrocytes was examined as inhibition of caspase-3 suppressed apoptosis in a cell line of immortalized human chondrocytes [Nuttall et al., 2000]. Caspases are synthesized as inactive proenzymes that are processed in cells undergoing apoptosis by self-proteolysis and/or cleavage by another protease. The processed form of procaspase-3 consists of large (17–22 kDa) and small (10–12 kDa) subunits, which associate to form an active enzyme. Active caspase-3 found in cells undergoing apoptosis [Krajewska et al., 1997] consists of 17 and 11 kDa subunits that are derived from the 32 kDa proenzyme (procaspase-3) by cleavage at multiple aspartic acid sites. As shown in Figure 4, non-loaded chondrocytes (i.e., lanes 1 and 2) synthesized only the 32 kDa procaspase-3. HP (5 MPa) increased procaspase-3 protein synthesis over the 4 h period. (Fig. 4, lanes 4–6). This was accompanied by the appearance of immunoreactive activated caspase-3 (17 kDa). Studies conducted on two independent chondrocyte strains (Fig. 4, lanes 1–6) produced similar results. The appearance of activated caspase-3 (Fig. 4) preceded the first indication of the 89 kDa PARP cleavage product at 4 h (Fig. 3).

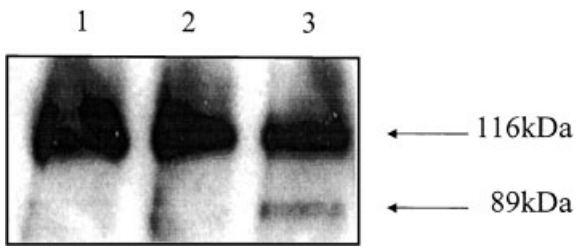

Fig. 3. Western blot. PARP degradation. An anti-PARP antibody (purified mouse anti-human PARP; clone 7D3-6; PharMingen International) was employed to determine the effect of HP (5 MPa) on PARP degradation. **Lane 1**: non-loaded cells; **lane 2**, HP, 5 MPa, 2 h; **lane 3**, HP, 5 MPa, 4 h. The immunoreactive 89 kDa PARP fragment and intact immunoreactive PARP (116 kDa) are indicated by arrows.

HP Induces *p53, c-myc,* and *bax-α* and Suppresses *bcl-2* mRNA

Total chondrocyte RNA and RT-PCR was employed to amplify the following gene transcripts with the expected size of amplified cDNA: *p53* (431 bp), *c-myc* (345 bp), *bcl-2* (293 bp), *bax-α* (365 bp), *ICAM-3* (555 bp), *p21/WAF*

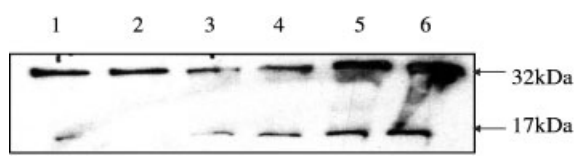

Fig. 4. Western blot. Synthesis and activation of procaspase-3. An anti-caspase-3 antibody (anti-CPP32, clone 19; Transduction Laboratories) was employed to determine the effect of HP (5 MPa) on procaspase-3 protein (32 kDa) and activated caspase-3 protein (17 kDa). **Lanes 1** and **2**: non-loaded chondrocytes (donor-1, donor-2); **lanes 3** and **4**, HP (5 MPa, 2 h, donor-1, donor-2); **lanes 5** and **6**, HP (5 MPa, 4 h, donor-1, donor-2).

(561 bp), *bcl-x* (353 bp), *c-fos* (431 bp), and *β-actin* (514 bp). The results of RT-PCR shown in Figure 5A indicated that HP (5 MPa for 4 h) induced *c-myc*, *p53*, and *bax-α* mRNA expression. Several other genes, including, *c-fos*, *ICAM-3*, and *p21/WAF* were expressed in both non-loaded and pressure-loaded chondrocytes, while *bcl-x* gene expression was not detected either in non-loaded or pressure-loaded cells (data not shown). By contrast, HP suppressed *bcl-2* mRNA when chondrocytes were subjected to HP for 2 h at 5 MPa but not at 2.5 MPa for 2 h (Fig. 5B). HP did not alter β-actin mRNA (Fig. 5C). Chondrocytes derived from two independent donor cartilage samples produced identical results (Fig. 5C, lanes 1–5).

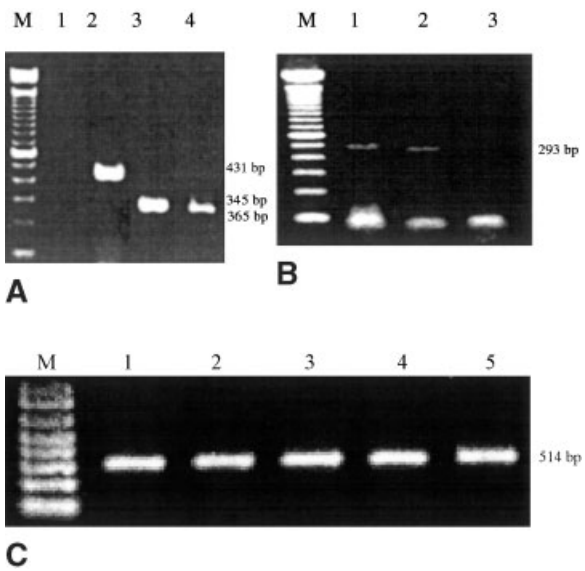

Fig. 5. RT-PCR. HP induces *p53*, *c-myc*, and *bax-α* expression and suppresses *bcl-2* expression. **Panel A**: *p53*, *c-myc*, and *bax-α* RT-PCR. The amplification products for *p53*, *c-myc*, and *bax-α* are shown. **Lane M**: DNA ladder; **lane 1**, non-loaded chondrocytes, amplification of *p53*. The cDNA from non-loaded chondrocytes was also subjected to RT-PCR to amplify *c-myc* and *bax-α* cDNA. No amplification products were detected for either of these cDNAs; **lane 2**, HP (5 MPa, 4 h), amplification of *p53*; **lane 3**, HP (5 MPa, 4 h), amplification of *c-myc*; **lane 4**, HP (5 MPa, 4 h) amplification of *bax-α*. **Panel B**: *Bcl-2* RT-PCR. The amplification product for *bcl-2* is shown. RT-PCR of cDNA from non-loaded and chondrocytes subjected to HP. **Lane M**: DNA ladder; **lane 1**, non-loaded chondrocytes; **lane 2**, HP, 2.5 MPa, 2 h; **lane 3**, HP, 5 MPa, 2 h. **Panel C**: *β-actin* RT-PCR. The amplification product for β-actin is shown. **Lane M**: DNA ladder; **lane 1**, non-loaded chondrocytes [donor-1]; **lane 2**, HP, 5 MPa, 2 h [donor-1]; **lane 3**, HP, 5 MPa, 2 h [donor-2]; **lane 4**, HP, 5 MPa, 4 h [donor-1]; **lane 5**, HP, 5 MPa, 4 h [donor-2].

Quantitative Real-Time RT-PCR

Quantitative real-time RT-PCR was employed to confirm the apparent suppression of *bcl-2* gene expression by HP and to determine whether other pro-apoptotic genes, namely, *TNF-α*, and *iNOS* were also affected by HP. Total RNA was isolated from chondrocytes subjected to HP or non-loaded chondrocytes and analyzed for the human housekeeping *R18* gene as well as *bcl-2*, *TNF-α*, and *iNOS*. The mRNA copy number for *bcl-2*, *TNF-α*, and *iNOS* was normalized to the R18 mRNA copy number in each sample. As is evident from Figure 6, HP at 5 MPa resulted in a *bcl-2* mRNA copy number, which was decreased by 1.3 and 2.6 log-fold after 2 and 4 h, respectively (Fig. 6). In contrast, a 1.9 and 2.5 log-fold increase in *TNF-α* gene expression was seen after HP for 2 h and 4 h, respectively. The *iNOS* gene was similarly affected (Fig. 6). *iNOS* expression was found to increase by ~1.9 and 2.4 log-fold when chondrocytes subjected to HP were compared to non-loaded controls after 2 and 4 h, respectively (Fig. 6).

Western Blots

The results of *p53*, *c-myc*, and *bcl-2* RT-PCR were confirmed by Western blots. HP (5 MPa) induced p53 protein (53 kDa) (Fig. 7A). The monoclonal antibody employed for the immunodetection of p53 protein does not distinguish between wild-type and mutant p53. HP also

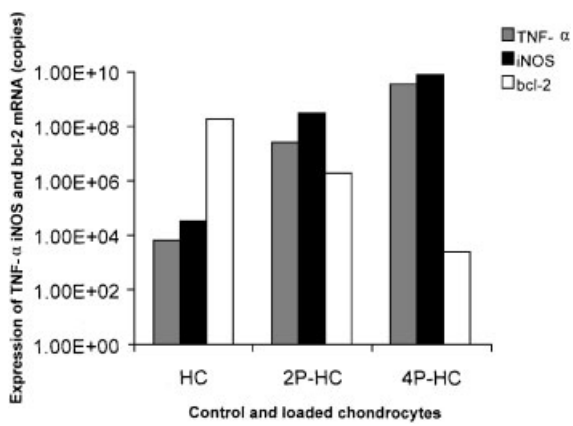

Fig. 6. Real-time quantitative RT-PCR. Pressure loading upregulates *TNF-α* and *iNOS* mRNA and suppresses *bcl-2* mRNA copy number. HC, non-loaded chondrocytes; 2P-HC, 5 MPa, 2 h; 4P-HC, 5 MPa, 4 h. A representative of three independent experiments is shown. The log-fold changes in mRNA copy number was calculated using the conversion $2^{-(\Delta\Delta C_T)}$ which represents the cycle difference between gene expressions corrected for 18S.

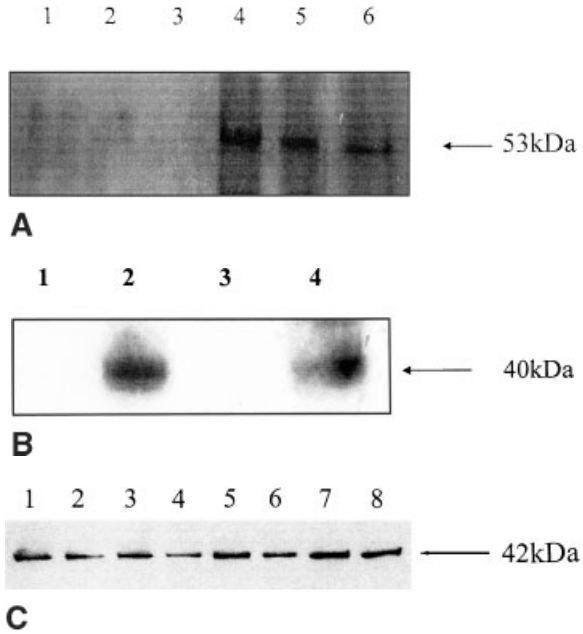

Fig. 7. Western blot. HP induces p53 and c-myc protein, but not β-actin protein. **Panel A**: p53 Western blot. A polyclonal antibody ([pan (polyclonal BMG-1B1)]; Boehringer Mannheim) reactive with p53 was employed to determine the effect of HP (5 MPa) on p53. **Lane 1**: non-loaded chondrocytes, 4 h; **lane 2**, non-loaded chondrocytes, 2 h; **lane 3**, non-loaded chondrocytes, 30 min; **lane 4**, HP, 4 h; **lane 5**, HP, 2 h; **lane 6**, HP, 30 min. The immunoreactive p53 protein (53 kDa) is indicated by the arrow. **Panel B**: c-myc Western blot. A monoclonal antibody (mouse anti-human c-myc, clone 9E10; Boehringer Mannheim) reactive with c-myc was employed to determine the effect of HP (5 MPa) on c-myc. **Lane 1**: non-loaded chondrocytes, 2 h; **lane 2**, HP, 2 h; **lane 3**, non-loaded chondrocytes, 4 h; **lane 4**, HP, 4 h. The major immunoreactive 40 kDa product is indicated by the arrow. **Panel C**: β-actin Western blot. A monoclonal antibody specific for various isoforms of β-actin (C-2; Santa Cruz Biotechnology) was employed to determine the effect of HP (5 MPa) on β-actin. **Lanes 1** and **2**: non-loaded chondrocytes from donor-1 [lane 1]; donor-2 [lane2]; HP, 30 min, donor-1 [lane 3]; donor-2 [lane 4]; HP, 2 h, donor-1 [**lane 5**]; donor-2 [**lane 6**]; **lanes 7** and **8**, HP, 4 h, donor-1 [lane 7]; donor-2 [lane 8]. The arrow shows the immunoreactive β-actin protein (42 kDa).

induced c-myc protein; major product, 40 kDa (Fig. 7B) and minor products, 150 and 44 kDa (data not shown). β-actin protein was detected in non-loaded as well as chondrocytes subjected to HP (Fig. 7C). There was no significant change in the intensity of the immunoreactive β-actin band (42 kDa) in pressure-loaded (5 MPa) chondrocytes after 30 min and 2 h. However, there was some indication that HP increased immunoreactive β-actin, after 4 h. (Fig. 7C, lanes 7 and 8). Chondrocyte strains derived from 2 independent donors produced similar results (Fig. 7C, lanes 1–8). In contrast to the effect of HP on p53 and c-myc protein, HP

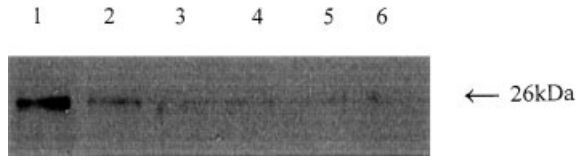

Fig. 8. Western blot. HP suppresses bcl-2 protein. A monoclonal antibody reactive with bcl-2 (mouse anti-human bcl-2, Ab-1; Oncogene Research Products) was employed to determine the effect of HP on bcl-2. **Lane 1**: non-loaded chondrocytes; **lane 2**, HP, 2.5 MPa, 4 h; **lane 3**, HP, 5 MPa, 2 h; **lane 4**, HP, 5 MPa, 4 h [donor-1]; **lane 5**, HP, 5 MPa, 2 h [donor-2]; **lane 6**, HP, 5 MPa, 4 h [donor-2]. The arrow shows the immunoreactive bcl-2 product (26 kDa).

(5 MPa) decreased bcl-2 protein (26 kDa), which was dependent on the magnitude of pressure and loading time (Fig. 8).

DISCUSSION

Mechanical signals regulate the growth, development, and function of a variety of tissues. Articular cartilage, a specialized load-bearing connective tissue, with outstanding friction, lubrication, and wear properties responds to mechanical pressure [Mow et al., 1999]. During locomotion, articular cartilage is subjected to dynamic loading, a major component of which is perpendicular to the articulating surfaces. Chondrocytes are excellent sensors of mechanical, ionic, and osmotic signals and respond to these signals in coordination with other environmental, hormonal, and genetic factors to regulate metabolic activity. Chondrocytes like many other cell types detect and respond to the applied load by altering their metabolic state through a process known as mechanotransduction [French, 1992; Ko and McCulloch, 2001]. This likely provides a mechanism by which chondrocytes quantitatively modulate rates of extracellular matrix synthesis and degradation. Mechanotransducing pathways which regulate apoptosis in cartilage have been largely unexplored in human chondrocytes in vitro, although a recent report provided morphological evidence of apoptosis in mature bovine patellar groove cartilage explants with loads above 6.5 MPa [Bee et al., 2000].

The "resident" cartilage from osteofemoral heads was employed in this study. Animal models of OA have shown that the articular cartilage and matrix metalloproteinase activity in sites topographically remote from the site of specific cartilage ulcers was altered when compared to control tissues [Malemud et al., 1986]. Nevertheless, the "resident" human OA

cartilage, which provided the chondrocytes employed in these studies showed no evidence of apoptosis unless HP was applied in vitro. This result supports recent studies, which showed that the frequency of apoptosis in OA cartilage is quite low [Aigner et al., 2001] and is dependent on tissue sampling. Thus, the "resident" cartilage from OA femoral heads is topographically remote from the regions of highest load in vivo and may resist apoptosis unless load is applied in vitro. The results of the present experiments support such a concept.

The results of this study provided compelling evidence that cyclic continuous HP employing a load magnitude and frequency associated with normal gait reduced chondrocyte viability and induced apoptosis. HP reduced chondrocyte viability at high initial starting densities when compared to lower initial densities (Table II). Previous findings demonstrated that maintenance of chondrocytes at high density (i.e., 5×10^5 to 10^6 cells ml^{-1}) correlated with the appropriate repertoire of newly synthesized proteoglycans and Type II collagen (COL2A1) gene expression [Malemud and Papay, 1984; Ganu et al., 1994; Malemud et al., 1995]. The reduction in chondrocyte viability induced by HP at 30 min occurred prior to any evidence of DNA fragmentation (Fig. 2) suggesting that early chondrocyte responses to HP may result from chondrocyte activation well prior to changes in nuclear events which result in DNA degradation. In support of this, DNA fragmentation typical of apoptosis, was seen after 2 and 4 h of HP (Fig. 2) and PARP degradation was only detected after 4 h of HP (Fig. 3).

Recent studies have indicated that several genes play a critical role in initiating the apoptotic process [reviewed in O'Connor and Strasser, 1999]. HP induced *p53* and *c-myc* expression. Overexpression of the early response gene, *c-myc* can result in *p53* gene expression and activation [Levine, 1997]. It is noteworthy that when p53 is expressed at high levels, other pathways also involved in apoptosis may be activated which could alter the up- or down-regulation of genes or interaction of *p53* with other proteins [Levine, 1997]. Thus, RT-PCR also revealed that HP induced *bax-α* gene expression. In previous studies, p53 was shown to affect *bax*, an antagonist of *bcl-2* [Prisco et al., 1997]. With respect to cartilage repair pathways, *p53* has also been shown to alter insulin-like growth factor-I (IGF-I) receptor synthesis and one of its binding proteins, IGF-BP3 [Buckbinder et al., 1995]. Further, the protein kinase R-activating protein (PACT) and protein kinase R (PKR) are upstream components of signaling pathways dependent, in part, on *c-myc* and *c-fos*. In the present study, HP did not induce *c-fos*. Nevertheless, recent studies [Gilbert et al., 2002] have shown that PACT and PKR are found in bovine and human OA cartilage and that TNF-α increased phosphorylation of PKR, which in turn, mediated TNF-α-induced activation of transcription factor, NF-κB [F. Chen et al., 2001].

In contrast to up-regulation of *c-myc*, *p53*, and *bax-α* by HP, *bcl-2* mRNA was markedly reduced. The importance of *bcl-2* suppression is underscored by the finding that if *bcl-2* is overexpressed it can provide short-term protection against apoptosis, and may even increase long-term cell survival [reviewed in O'Connor and Strasser, 1999]. Thus, it is noteworthy that human OA chondrocytes expressed both the *bcl-2* gene and bcl-2 protein in the non-loaded state but *bcl-2* gene expression was suppressed several log-fold and bcl-2 protein levels apparently reduced by HP. The undetectable bcl-2 protein (Fig. 8, lanes 5 and 6) may have resulted from the low-yield *bcl-2* mRNA copy number measured by quantitative real-time RT-PCR (Fig. 6). Thus, apoptosis induction by HP was likely to be directly related to *bcl-2* suppression. Although the specific mechanism resulting in bcl-2 suppression in response to HP is unknown, previous studies have shown that chondrocyte apoptosis is linked to structural changes in mitochondrial membranes and low levels of bcl-2 [Hashimoto et al., 1997; Feng et al., 1998] although other mechanisms must also be considered as bcl-2 cleavage by activated caspases [Tomicic and Kaina, 2001] also promotes apoptosis.

HP up-regulation of pro-apoptotic and suppression of anti-apoptotic gene expression also resulted in parallel changes in protein synthesis as evidenced by Western blotting. β-actin was found in non-loaded chondrocytes as well as chondrocytes subjected to HP (Fig. 7C) indicating that lanes in the Western blots for p53, c-myc, and bcl-2 for which no immunoreactive product was detected (Figs. 7 and 8) contained protein, but in these lanes the specific proteins (i.e., p53, c-myc, bcl-2) were below the detection level to be reactive with the specific antibody employed.

While HP resulted in low magnitude bcl-2 protein levels at 2.5 MPa, this was not the case when HP at 5 MPa was employed. This pressure level was also required for induction of apoptosis. For example, PARP degradation (Fig. 3) as well as internucleosomal DNA fragmentation readily identified when 5 MPa was employed was not detected at 2.5 MPa (data not shown). Further, synthesis and activation of caspase-3 protein also was time-dependent, suggesting that caspase-3 activation mediated pressure-induced apoptosis and reduced cell survival. We had previously determined [Islam et al., 2000] that a synthetic pan-caspase inhibitor (Z-VAD-FMK) and a caspase-3-specific inhibitor (DEVD) prevented apoptosis induced by epigallocatechin-3-gallate in a human chondrosarcoma cell line (HTB-94), indicating that apoptosis in this chondrocyte-like cell line was dependent on activated caspase-3.

TNF-α and *iNOS* gene expression was previously identified in normal human cartilage with significant up-regulation associated with OA [Attur et al., 2002]. Thus, the results in Figure 6 showing *TNF-α* and *iNOS* gene expression in non-loaded OA chondrocytes was not unexpected. However, HP upregulated the *TNF-α* and *iNOS* genes by several log-fold as evidenced by real-time quantitative RT-PCR. In this case, up-regulation of *TNF-α* and *iNOS* was associated with induction of apoptosis.

Recent studies showed TNF-α to induce apoptosis in both non-hypertrophic and hypertrophic chondrocytes, which was characterized by positive immunostaining for the Fas receptor and caspase-2 [Aizawa et al., 2001]. Evidence also suggests that human chondrocytes express both p55 and p75 TNF receptors [Westacott et al., 1994] and synoviocytes and chondrocytes derived from OA joints showed increased TNF receptors compared to their normal counterparts [Alaaeddine et al., 1997; Webb et al., 1997]. In addition, culture supernates from OA synovium or synovial fluid from OA joints apparently resulted in increased TNF receptors on normal chondrocytes when these synovial fluids were added in vitro [Webb et al., 1998]. It is now understood that the p55 TNF receptor and the Fas receptor share a common signaling pathway through which specific proteins such as TRADD and FADD activate the proteolytic cascade resulting in apoptosis [Chinnaiyan et al., 1995; Muzio et al., 1996]. It is noteworthy that preliminary experiments employing an anti-FADD monoclonal antibody (mouse anti-human FADD clone A66-2; PharMingen International) and Western blotting also showed an apparent increase in FADD protein (i.e., a 48 kDa form and expected 26 kDa form) in response to HP (5 MPa) which was seen after 1 h and was sustained to the 4 h time point (data not shown).

Cartilage erosions appear to correlate topographically with sites rich in apoptotic cells [Erlacher et al., 1995]. The results of the present study suggest that, in part, HP induced apoptosis by increasing OA chondrocyte *TNF-α* gene expression, which may subserve HP in initiating apoptosis. Thus, these results support the view that TNF-α autoamplification could exert a strong pro-apoptosis effect in human OA cartilage. Up-regulation of TNF-α as a mediator of apoptosis in human cartilage may be particularly significant when cartilage extracellular matrix has been mechanically or enzymatically disrupted [Fischer et al., 2000], as so often occurs in human OA cartilage. Further, a pro-apoptosis amplification pathway could also be further accelerated as HP also upregulated iNOS by several log-fold. NO has been shown not only to induce apoptosis in chondrocytes [Hashimoto et al., 1998; Pelletier et al., 1998; Notoya et al., 2000] and to be upregulated in OA cartilage [Attur et al., 2002], but can also cause activation of metalloproteinases in articular cartilage as well [Murrell et al., 1995].

Finally, since load affects cartilage proteoglycan synthesis in vitro, it is possible that the effects of HP on apoptosis also affect proteoglycan synthesis [Parkkinen et al., 1993b; Lammi et al., 1994] by inducing changes in viable cells via the products released by apoptotic cells. Unlike many other tissues in which apoptosis has been measured, articular cartilage is aneural and avascular. How the final disposition of apoptotic bodies from chondrocytes is achieved remains to be determined.

The novel findings of the present study could shed light on the complex regulatory networks in human chondrocytes by which up-regulation of *p53*, *c-myc*, and *bax-α* which are pro-apoptotic are balanced by anti-apoptotic expression of *bcl-2* and *bcl-2* family members. While it remains to be determined whether non-arthritic chondrocytes age-matched to samples derived from human osteofemoral head cartilage undergo apoptotic changes in response to HP, preliminary studies employing chondrocytes

derived from "macroscopically normal" human knee cartilage suggested that these cells are not apoptosis-resistant as TNF-α and sodium nitroprusside induced apoptotic changes similar to those found in OA chondrocytes [Haqqi et al., unpublished data]. Thus, an imbalance between pro-apoptotic and anti-apoptotic pathways may be just as important in the development of cartilage alterations in OA as is the imbalance between metalloproteinases and their inhibitors which are known to play a prominent role in OA pathology [Malemud and Goldberg, 1999].

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