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Regulation of Nitric Oxide and *bcl-2* Expression by Shear Stress in Human Osteoarthritic Chondrocytes In Vitro

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Abstract Onset and progression of cartilage degeneration is associated with shear stress occurring in diarthrodial joints subjected to inappropriate loading. This study tested the hypothesis that shear stress induced nitric oxide is associated with altered expression of regulatory onco-proteins, *bcl-2*, and Fas (APO-1/CD95) and apoptosis in primary human osteoarthritic chondrocyte cultures. Shear stress induced membrane phosphatidylserine and nucleosomal degradation were taken as evidence of chondrocyte apoptosis. Application of shear stress upregulated nitric oxide in a dose-dependent manner and was associated with increases in membrane phosphatidylserine and nucleosomal degradation. Increasing levels of shear stress decreased expression of the anti-apoptotic factor, *bcl-2*, from 44 to 10 U/ml. Addition of the nitric oxide antagonists, L-N^5-(1-iminoethyl) ornithine and Nω-nitro-L-arginine methyl ester (L-NAME), reduced shear stress induced nucleosomal degradation by 62% and 74%, respectively. Inhibition of shear stress induced nitric oxide release by L-NAME coincided with a 2.7-fold increase of *bcl-2*, when compared to chondrocytes exposed to shear stress in the absence of L-NAME. These data suggest that shear stress induced nitric oxide is associated with changes in apoptotic regulatory factors that alter chondrocyte metabolism and may contribute to joint degeneration. J. Cell. Biochem. 90: 80–86, 2003. Published 2003 Wiley-Liss, Inc.†

Key words: articular chondrocytes; osteoarthritis; shear stress; human; nitric oxide; apoptosis; Fas; *bcl-2*

Diarthrodial joints are subjected to a variety of mechanical loading conditions that influence articular cartilage in homeostasis and disease [Radin et al., 1991]. Within the joint, chondrocytes are exposed to either dilatational (compressive hydrostatic pressure) or deviatoric stresses such as shear stress [Carter and Wong, 1990]. A number of studies associate shear stress with release of proinflammatory mediators [Mohtai et al., 1996; Trindade et al., 2001] and development of arthritis and bone destruction [Moskowitz et al., 1981; Stockwell et al., 1983; Bogoch and Moran, 1999]. Shear stress and proinflammatory cytokines induce heat shock proteins in synovial fibroblasts isolated from patients with rheumatoid arthritis [Schett et al., 1998].

Osteoarthritic cartilage exhibits histomorphological evidence of nuclear clumping, empty lacunae, loss of intracellular organelles, and matrix integrity [Weiss and Mirow, 1972]. Immunocytochemical, flow cytometric, and electron microscopic analyses reveal that up to 51% of osteoarthritic chondrocytes are apoptotic [Blanco et al., 1998]. Unlike necrosis, apoptosis is an energy dependent process involving cell shrinkage, plasma membrane blebbing, nuclear condensation, intact cell membranes, and controlled autodigestion of cells [Cohen, 1993; Thompson, 1995]. Recent evidence suggests that alterations in cell survival contribute to the pathogenesis of a variety of human diseases and that regulation of apoptosis may provide a

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target for therapeutic intervention [Miller and Marx, 1998].

Nitric oxide, a potent inducer of apoptosis in musculoskeletal system [Blanco et al., 1995; Damoulis and Hauschka, 1997], is directly linked to the pathogenesis of arthritis [Stefanovic-Racic et al., 1993; Evans et al., 1995; Murrel et al., 1995]. Carter and Wong [1990] propose that shear stress influences the processes of degeneration and ossification of articular cartilage that continues throughout life. Previous studies demonstrate that shear stress induces nitric oxide release in normal bovine articular chondrocytes and human osteoarthritic chondrocytes [Das et al., 1997; Smith et al., 2000]. This study examined the hypothesis that shear stress induced nitric oxide alters expression of the regulatory onco-proteins, *bcl-2*, and Fas (APO-1/CD95) and processes associated with apoptosis in primary human osteoarthritic chondrocytes.

MATERIALS AND METHODS

Cell Culture

Osteoarthritic cartilage specimens were collected following total joint arthroplasty from regions of the joint surface without visible softening and fraying indicative of moderate to severe fibrillation. The chondrocytes were dissociated at 37°C in 15 ml of Dulbecco's modified Eagle medium [(DMEM), GIBCO, Grand Island, NY] containing 10% fetal bovine serum (FBS), 25 μg/ml gentamicin, and 1 mg/ml each Class II and IV bacterial collagenase (Worthington Chemical, Freehold, NJ). The cells were washed two times in Dulbecco's phosphate buffered saline without Mg^{2+} and Ca^{2+}, washed once with DMEM, and then plated at 2.6×10^4 cells/cm^2 in DMEM containing FBS, gentamicin, and ascorbic acid (25 μg/ml) (Sigma Chemical, St. Louis, MO). High density monolayer cultures were tested in serum-free medium containing 25 μg/ml gentamicin, 25 μg/ml ascorbic acid, 1 mM selenium, and a liposome supplement [Jones and Smith, 1990].

Application of Shear Stress

Shear stress was applied using a cone viscometer modified from Bussolari et al. [1982] as previously described [Mohtai et al., 1996; Das et al., 1997]. Briefly, the cultures were exposed to shear stress at rotating velocities of 20, 50, 100, or 200 rpm for 2 h. Speed was controlled by a servo-electronic feedback circuit and corresponds to shear stress levels of 0.16, 0.41, 0.82, and 1.64 Pa, respectively. Under these loading conditions, the culture medium exhibits a predominantly concentric flow, accompanied by a radial secondary flow with no turbulence. Control cells were not exposed to shear stress but were maintained under the identical culture conditions. After loading, the cells were maintained under the same culture conditions for a constant period of 24 h to allow post-loading cellular processing. Each experiment was performed in triplicate and repeated with individual cell preparations isolated from three to five cartilage specimens. In some experiments, nitric oxide synthase inhibitors, Nω-nitro-L-arginine methyl ester (L-NAME, 1 mM) and L-N^5-(1-iminoethyl) ornithine (NIO, 1 mM) (Sigma) were added to the cultures.

Quantification of Nitric Oxide Release

The concentration of nitrite, the stable end product of nitric oxide oxidation, was used as an indicator of nitric oxide synthesis. Nitrite concentration in the culture medium was measured spectrophotometrically using the Griess reaction with sodium nitrite as the standard [Green et al., 1982]. Briefly, an aliquot (100 μl) of collected culture medium was incubated with 50 μl of a 0.1% solution of sulfanilamide in 5% phosphoric acid and 50 μl of a 0.1% solution of N-1-naphthyl-ethylenediamine dihydrochloride (Sigma) for 10 min for measurement of absorbance at 550 nm.

Detection of Apoptosis

Chondrocytes were cultured on coverslips and subjected to shear stress as described above. Levels of apoptosis were determined based on phosphatidylserine expression as detected by annexin V-FITC labeling (Annexin V-FITC Apoptosis Detection Kit, Oncogene, Cambridge, MA) using confocal microscopy (Nikon Eclipse TE300, MRC-1024 Laser Scanning Confocal Imaging System, BioRad). Levels of apoptosis were also quantified based on nucleosomal cleavage of DNA as detected by a commercially available nucleosome ELISA (Oncogene). Briefly, cell lysates were added to a precoated ELISA plate containing DNA binding protein. Anti-histone biotin-labeled antibodies then recognized the histone component of captured nucleosomes and were detected following incubation with a streptavidin-linked horseradish

peroxidase conjugate and chromagen, tetramethylbenzidine (TMB).

Quantification of *bcl-2* and Fas Expression

Assays for *bcl-2* proto-oncogene and Fas antigen were carried out using commercially available kits (*bcl-2* ELISA and Fas/APO-1 ELISA, Oncogene) according to the manufacturer's instructions.

Statistical Analysis

One-way analysis of variance (ANOVA) with post-hoc Newman-Keuls multiple comparisons was used for statistical comparisons with $P < 0.05$ considered to be significant.

RESULTS

Externalization of phosphatidylserine to the outer plasma membrane is an indicator of apoptosis that precedes DNA fragmentation and nuclear breakdown. Exposure of human osteoarthritic chondrocytes to shear stress for 2 h followed by a 24-h culture period induced translocation of phosphatidylserine (PS), as detected by fluorescein isothiocyanate (FITC) conjugated annexin V. Translocation of phosphatidylserine increased in dose-dependent manner as the shear stress increased (0.16–0.82 Pa) by increasing the viscometer speed (20–100 rpm) (Fig. 1).

Endonuclease-mediated digestion of the DNA linker regions between nucleosomes in chromatin is a second indicator of activation of apoptotic processes. With increasing levels of shear stress, nucleosomal DNA fragments increased to 0.7, 5.7, 9.6, and 38.7 U/ml at 20, 50, 100, and 200 rpm (ANOVA, $P < 0.001$), respectively (Fig. 2A). Nucleosomal degradation was not observed in chondrocytes in the absence of shear stress. Expression of the proto-oncogene *bcl-2*, an inhibitor of apoptosis, decreased from 44 U/ml in control samples to 40, 28, 26, and 10 U/ml with the rotating velocities of 20, 50, 100, and 200 rpm (ANOVA, $P < 0.001$), respectively (Fig. 2B).

Nitrite levels in the culture medium increased as the rotational velocity increased in a dose-dependent manner (ANOVA, $P < 0.001$), coinciding with increased apoptosis and down-regulation of *bcl-2* expression (Fig. 2C). The

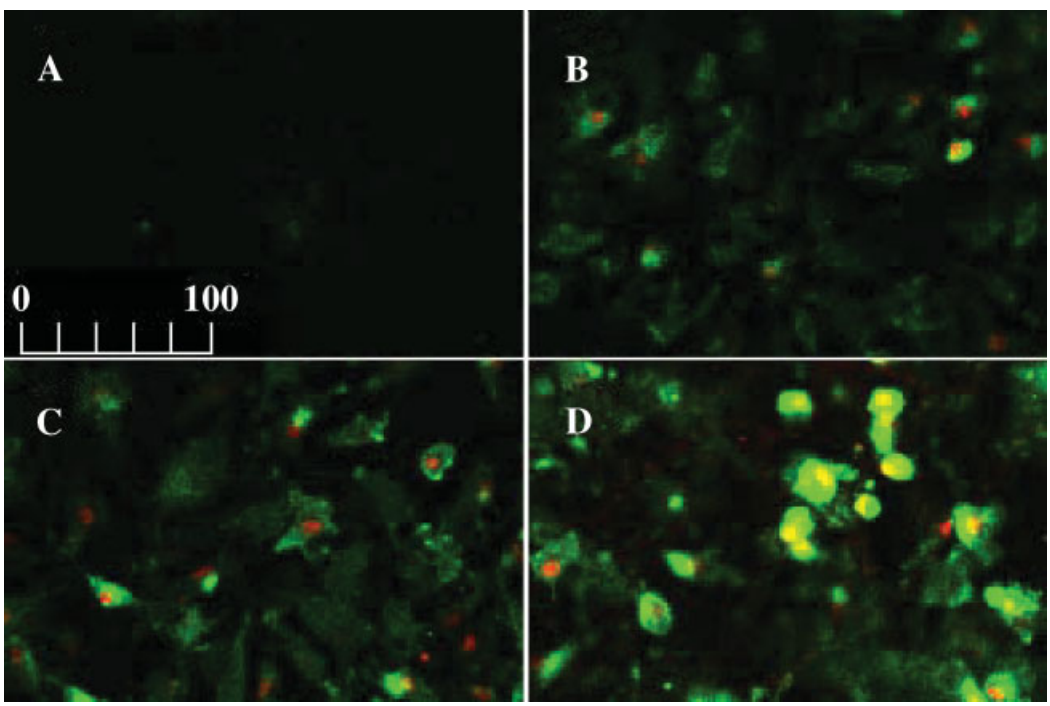

Fig. 1. Induction of osteoarthritic chondrocyte apoptosis by shear stress. In the absence of shear stress (**A**), chondrocytes did not exhibit apoptosis as evidenced by Annexin V-FITC/propidium iodide double staining. Chondrocytes exposed to increasing levels of shear stress (20 rpm) (**B**) for 2 h and tested after 24 h of culture showed low levels of phosphatidylserine staining. At 50 rpm (**C**) and 100 rpm (**D**), chondrocytes exhibited progressively more phosphatidylserine staining. (Bar = 100 μm.) Each Figure is representative of five individual experiments.

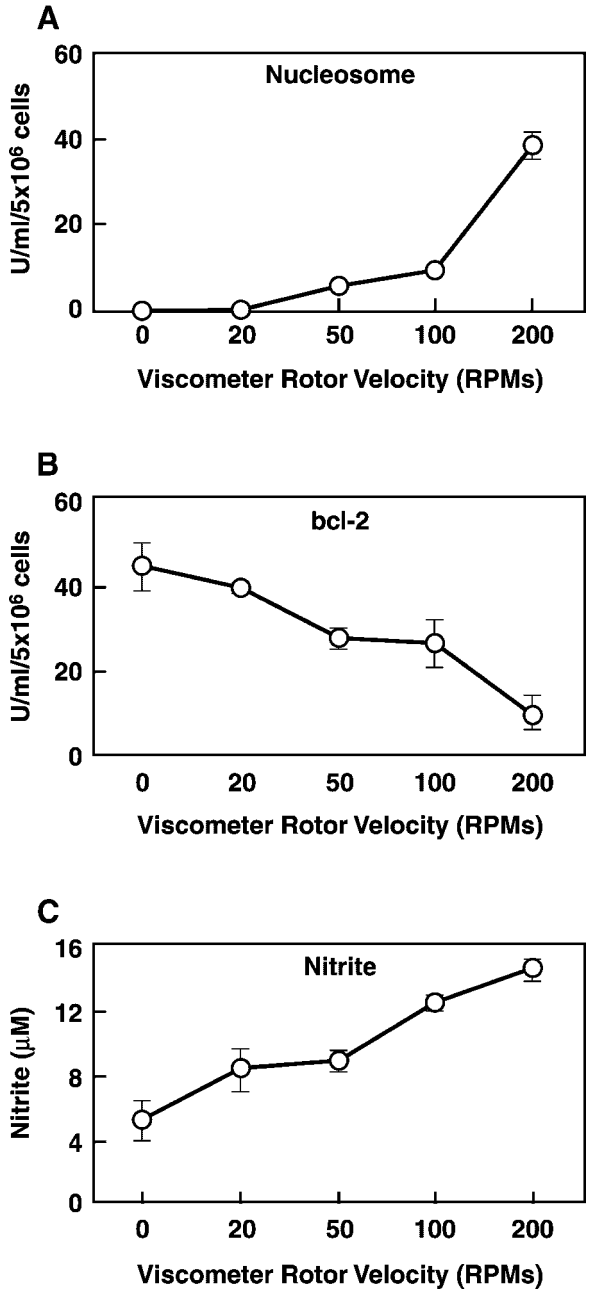

Fig. 2. Kinetic changes in apoptotic markers and nitric oxide in response to shear stress. With increased viscometer velocity (20–200 rpm), nucleosome levels increased from 0.7 to 38.7 (U/ml/5 × 10^6 cells) (**A**), *bcl-2* levels decreased from 40–to 10 (U/ml/5 × 10^6 cells) (**B**), and nitric oxide levels (represented by nitrite) increased from 8.3 to 14.4 μM (**C**). The data represent the mean and the standard error for individual cell preparations from five autopsy specimens.

nitric oxide inhibitors, L-N^5-(1-iminoethyl) ornithine (NIO) and Nω-nitro-L-arginine methyl ester (L-NAME), were added to test whether inhibition of nitric oxide production could limit the extent of chondrocyte apoptosis. The nitric oxide inhibitors were tested in chondrocyte cultures exposed to shear stress applied at a viscometer speed of 200 rpm (1.64 Pa) for 2 h followed by a 24-h culture period. Addition of NIO and L-NAME inhibited nucleosomal degradation by 62% and 74%, respectively, when compared to chondrocytes exposed to shear stress in the absence of inhibitors (ANOVA, $P < 0.001$) (Fig. 3A). Addition of NIO and L-NAME inhibited nitric oxide release in the chondrocytes in a similar pattern to that observed for the induction of apoptosis (Fig. 3B).

Cellular extracts from chondrocytes exposed to shear stress in the presence and absence of the nitric oxide inhibitor, L-NAME, were then subjected to the analysis of *bcl-2* and Fas (APO-1/CD95) expression. Addition of L-NAME to chondrocytes exposed to shear stress resulted in a 2.7-fold increase in the anti-apoptotic oncoprotein, *bcl-2*, relative to chondrocyte exposed

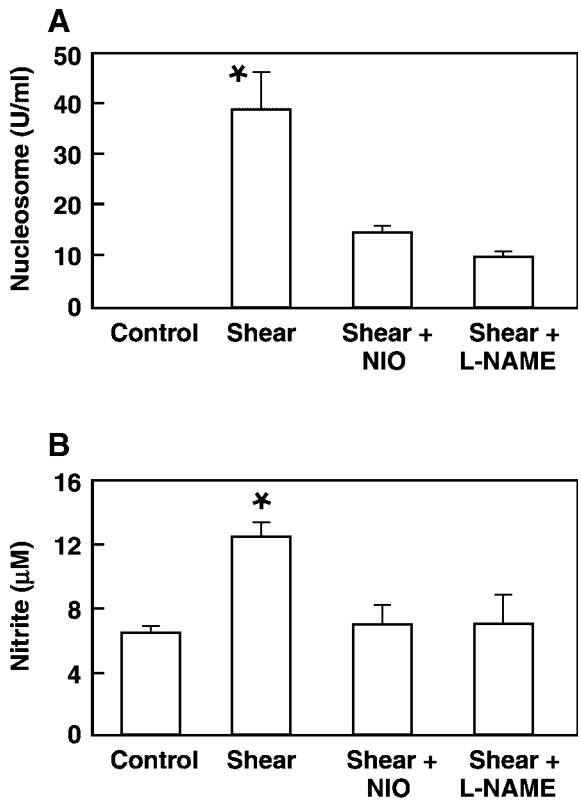

Fig. 3. Inhibition of shear stress induced osteoarthritic chondrocyte apoptosis by the nitric oxide inhibitors, L-N^5-(1-iminoethyl) ornithine (NIO) and Nω-nitro-L-arginine methyl ester (L-NAME). Nucleosome levels (**A**) and nitrite levels (**B**) were determined from samples in the presence and absence of NIO (1 mM) or L-NAME (1 mM). * denotes a significance level of $P < 0.001$. The data represent the mean and standard error for individual cell preparations from five cartilage samples.

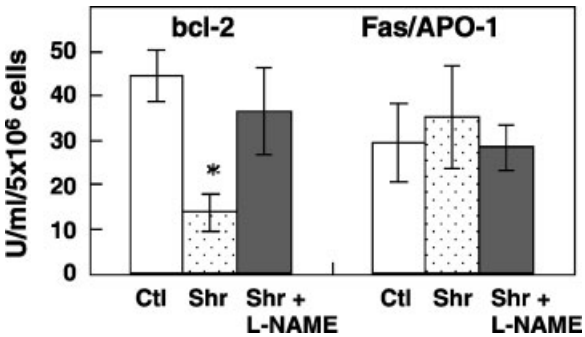

Fig. 4. Expression of *bcl-2* and Fas/CD95 in chondrocytes exposed to shear stress (200 rpm) with or without the nitric oxide inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME). Ctl denotes chondrocytes not exposed to shear stress; Shr denotes chondrocytes exposed to shear stress; Shr + L-NAME denotes chondrocytes exposed to shear stress in the presence of the nitric oxide inhibitor. * denotes significance at the level of $P < 0.001$ as compared to unsheared control (Ctl, open box) and shear with nitric oxide inhibitor (shear + L-NAME, solid box). The data represent the mean and standard deviation for individual cell preparations from three cartilage samples.

to shear stress in the absence of inhibitor (Fig. 4). Under the same conditions, Fas (APO-1/CD95) expression was unchanged in the chondrocytes with or without exposure to shear stress (Fig. 4).

DISCUSSION

Excessive or inappropriate mechanical force imparted to articular cartilage has been implicated as a contributing factor in arthroses [Thompson and Bassett, 1970; Simon, 1978; Carter et al., 1987]. Mechanically induced joint degeneration involves alterations in proteoglycan content [Rostand et al., 1986; Säämänen et al., 1990], thinning and fibrillation of the articular surface [Lukoschek et al., 1988], an imbalance between proteases and protease inhibitors [Ehrlich et al., 1977; Ehrlich, 1985; Dean et al., 1989], and induction of pro-inflammatory cytokines [Mohtai et al., 1996; Shinmei and Nemoto, 1996]. The results presented here show that shear stress dose-dependently upregulated nitric oxide release and altered processes associated with apoptosis in human osteoarthritic chondrocytes. Inhibition of nitric oxide production deceased evidence of shear stress induced apoptosis.

Nitric oxide is associated with human chondrocyte apoptosis [Stefanovic-Racic et al., 1993] and elevated levels of nitric oxide occur in serum and synovial fluid of arthritic patients [Farrell et al., 1992; Evans et al., 1995; Murrel et al., 1995] and in animals models of arthritis [Weinberg et al., 1994]. Inhibition of nitric oxide synthesis limits the severity of arthritis in animal models [McCartney-Francis et al., 1993; Stefanovic-Racic et al., 1994; Shyy et al., 1995].

In this study, the degree of activation of chondrocyte apoptosis was decreased but not eliminated by inhibiting shear stress induced nitric oxide release. These data suggest alternative mechanotransduction pathways, including effects on the cytoskeleton, potentiate chondrocyte apoptosis in addition to the effects of shear stress on nitric oxide release and apoptotic regulatory factor expression observed here. In vascular endothelial cells, shear stress increases nitric oxide release and regulates expression of platelet-derived growth factor, basic-fibroblast growth factor, transforming growth factor β-1, proto-oncogene products (c-Jun, c-Fos, and c-Myc), intracellular adhesion molecule-1, and monocyte chemotactic protein-1 [Adams and Cory, 1998]. In cultivated synovial cells from patients with rheumatoid arthritis, shear stress also induces a complete heat shock response including heat shock protein 70 and heat shock transcription factor [Schett et al., 1998]. Under certain conditions, other reactive oxygen species (ROS) may influence chondrocyte apoptosis with or without involvement of nitric oxide [Del Carlo and Loeser, 2002].

Bcl-2 is an anti-apoptotic onco-protein residing on the cytoplasmic face of the outer mitochondrial membrane, the endoplasmic reticulum, and the nuclear envelope. These intracellular membranes contain multiple cellular moieties (guanylyl cyclase, cyclo-oxygenases, aconitase, ribonucleotide reductase, membrane phospholipids, and DNA) with which nitric oxide may interact [Blanco et al., 1995; Shyy et al., 1995]. *Bcl-2* is also involved in regulation of chondrocyte apoptosis in the epiphyseal growth plate [Amling et al., 1997] and in osteoarthritic cartilage [Erlacher et al., 1995]. In this study, *bcl-2* expression decreased in association with induction of nitric oxide release and activation of apoptotic processes in osteoarthritic chondrocytes following exposure to shear stress. Inhibition of nitric oxide release prevented downregulation of *bcl-2* expression by shear stress. In contrast to effects of shear stress on *bcl-2,* expression of another mediator of chondrocyte apoptosis, Fas (CD95), was

unchanged in cells exposed to shear stress. These data are in agreement with the results of Hashimoto et al. [1997] that show similar levels of Fas in normal and osteoarthritic chondrocytes.

This study showed that shear stress activated processes associated with apoptosis in human osteoarthritic chondrocytes were linked to increased release of nitric oxide and decreased expression of *bcl-2*. These data implicate shear stress as an important element in inappropriate mechanical loads in causing degenerative changes in joint cartilage by influencing chondrocyte survival.

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