Evidence Linking Chondrocyte Lipid Peroxidation to Cartilage Matrix Protein Degradation

POSSIBLE ROLE IN CARTILAGE AGING AND THE PATHOGENESIS OF OSTEOARTHRITIS*

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Reactive oxygen species (ROS) are implicated in both cartilage aging and the pathogenesis of osteoarthritis. We developed an in vitro model to study the role of chondrocyte-derived ROS in cartilage matrix protein degradation. Matrix proteins in cultured primary articular chondrocytes were labeled with [3H]proline, and the washed cell matrix was returned to a serum-free balanced salt solution. Exposure to hydrogen peroxide resulted in oxidative damage to the cell matrix as established by monitoring the release of labeled material into the medium. Calcium ionophore treatment of chondrocytes, in a dose-dependent manner, significantly enhanced the release of labeled matrix, suggesting a chondrocyte-dependent mechanism of matrix degradation. Antioxidant enzymes such as catalase or superoxide dismutase did not influence matrix release by the calcium ionophore-activated chondrocytes. However, vitamin E, at physiological concentrations, significantly diminished the release of labeled matrix by activated chondrocytes. The fact that vitamin E is a chain-breaking antioxidant indicates that the mechanism of matrix degradation and release is mediated by the lipid peroxidation process. Lipid peroxidation was measured in chondrocytes loaded with cis-parinaric acid. Both resting and activated cells showed constitutive and enhanced levels of lipid peroxidation activity, which were significantly reduced in the presence of vitamin E. In an immunoblot analysis, malondialdehyde and hydroxynonenal adducts were observed in chondrocyte-matrix extracts, and the amount of adducts increased with calcium ionophore treatment. Furthermore, vitamin E diminished aldehyde-protein adduct formation in activated extracts, which suggests that vitamin E has an antioxidant role in preventing protein oxidation. This study provides in vitro evidence linking chondrocyte lipid peroxidation to cartilage matrix protein (collagen) oxidation and degradation and suggests that vitamin E has a preventive role. These observations indicate that chondrocyte lipid peroxidation may have a role in the pathogenesis of cartilage aging and osteoarthritis.

Cartilage degeneration is a hallmark of cartilage aging and osteoarthritis (1). Degeneration of articular cartilage in osteoarthritis is accompanied by chronic pain and significant disability. In a series of reports (2–7), we and others have documented that chondrocytes produce reactive oxygen species (ROS). The production of ROS by chondrocytes can contribute to degradation of the cartilage matrix. For example, ROS can mediate intracellular signaling and gene activation of cytokine and growth factor-induced products in chondrocytes (8, 9). In activated neutrophils and monocytes/macrophages, the cell-specific gene products of "NADPH-oxidase complex" physically come together and initiate single electron reduction of oxygen and the release of ROS outside the cells. Phagocytes use the toxic properties of ROS to eliminate pathogens (10, 11); in contrast, the biological role of secreted ROS in cartilage is not known.

The observation that in vitro exposure to ROS damages cartilage matrix suggests that chondrocyte-derived ROS may mediate matrix degradation (12–18). However, the in vitro role of ROS in cartilage matrix degradation is difficult to evaluate, because cartilage is avascular and ROS are extremely labile. Previously we reported that chondrocyte-derived hydrogen peroxide mediates aggrecan degradation, which can be inhibited by antioxidants (19). In the present study, we investigate the role of chondrocyte-derived ROS in cartilage matrix protein (collagen) degradation. Studying the mechanism of collagen degradation is important, because several studies indicate that damage to cartilage collagen is a central event in the pathogenesis of cartilage aging and osteoarthritis (20–23).

To determine the role of chondrocyte-ROS in cartilage matrix protein (collagen) degradation, we followed a paradigm used previously (11, 19) that has helped elucidate the functions of ROS in phagocytic cells. In these studies, phagocytic cells were often incubated with viable pathogens and cells were induced to yield oxidative bursts (11, 19). The role of ROS in killing pathogens is deciphered by using specific antioxidant enzymes or scavengers of ROS. In our model system, primary articular chondrocytes were cultured and collagen matrix was labeled with [3H]proline. Collagen is characterized by a triple helical structure of Gly-X-Y repeat sequence, where X and Y often are represented by proline and hydroxyproline. The major structural component of cartilage tissue is collagen type II. Incorpo-

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1 The abbreviations used are: ROS, reactive oxygen species; LPS, lipopolysaccharide from Escherichia coli 0127:B8; PMA, phorbol 12-myristate 13-acetate; fMLP, formylmethionylleucylphenylalanine; ConA, concanavalin A; SOD, superoxide dismutase; BHT, butylated hydroxytoluene; PG, propylgallate; Def, deferoxamine; HBSS, Earl’s balanced salt solution; EBSS, Earl’s balanced salt solution; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; TBS, Tris-buffered saline; MDA, malondialdehyde; HNE, hydroxynonenal; PUFAs, polyunsaturated fatty acids.
Chondrocyte Lipid Peroxidation and Cartilage Matrix Degradation

RATION OF RADIOLabeled PROLINE IS OFTEN USED TO STUDY THE SYNTHESIS AND DEGRADATION OF CARTILAGE COLLAGEN MATRIX (24, 25). ABOUT 40–70% OF LABELED MATERIAL IN THE MATRIX IS ASSOCIATED WITH COLLAGEN, THE REST IS ASSOCIATED WITH NONCOLLAGENOUS MATERIAL (26). THE RELEASE OF LABELED MATERIAL INTO THE MEDIUM FROM CELL-MONOLAYER-MATRIX CULTURES PROVIDES AN INDEX OF MATRIX DEGRADATION. USING THIS MODEL, WE OBSERVED THAT CHONDROCYTE ACTIVATION-DEPENDENT MATRIX DEGRADATION WAS MEDIATED BY LIPID PEROXIDATION AND NOT BY THE RELEASE OF ROS. WE ALSO DEMONSTRATED THE FORMATION OF ALDEHYDIC PROTEIN ADDUCTS WITH THE CARTILAGE MATRIX AND THE ROLE OF VITAMIN E. THE RELEVANCE OF THESE FINDINGS IN THE CONTEXT OF CARTILAGE AGING AND OSTEOARTHRITIS IS PRESENTED.

MATERIALS AND METHODS

Reagents—Lipopolysaccharide from Escherichia coli 0127:B8 (LPS), phorbol 12-myristate 13-acetate (PMA), formylmethionylleucylphenylalanine (fMLP), calcium ionophore A23187, 4-bromo-calcein ionophore A23187, concanavalin A (ConA), superoxide dismutase, catalase, N-t-butylphenylsulfonitron, ascorbic acid, vitamin E, butylated hydroxytoluene (BHT), propylgallate, and deferoxamine (Def) were purchased from Sigma (St. Louis, MO). Hydrogen peroxide of reagent grade was from Fisher Scientific (Fair Lawn, NJ). Dulbecco’s minimum essential medium, fetal bovine serum, Hanks’ balanced salt solution (HBSS), Earl’s balanced salt solution (EBSS), t-glutamine, gentamicin, HEPES buffer, penicillin, and streptomycin were purchased from Life Technologies, Inc. [3,3,4,4,5,5,5-H]Proline, with a specific activity of 1.6 GBq/mmol, was obtained from NEN Life Science Products.

Isolation of Rabbit Articular Chondrocytes—New Zealand White rabbits (2.7–3.6 kg) of either sex were killed by intravenous injection of Beuthanasia-D special (Schering Corp., Kenilworth, NJ). The chondrocytes were isolated as described previously (27). The viability of chondrocytes was confirmed by trypan blue exclusion. Primary chondrocytes were isolated as described previously (27). The viability of chondrocytes was confirmed by trypan blue exclusion.

Primary rabbit articular chondrocytes were distributed into 24-well plates at a concentration of 1–2 × 10⁶ cells/well in 1 ml of complete media. Chondrocytes were allowed to attach for 3–5 days, and media was changed every 3 days. Confluent cells in multwell plates were labeled with 1–2.5 μCi/well with [3H]Proline during the last 24–48 h of cell culture. The cell monolayer was washed at least four to five times with warm HBSS by figuring plates to remove unincorporated proline from the matrix. Albumin or serum-free EBSS was added to the wells. Experiments were carried out in triplicate wells. The test reagents were added, and the total volume was adjusted to 0.5 ml with EBSS. The cultures were incubated at 37 °C in a humidified 5% CO₂ incubator for 4–24 h. A 100-μl aliquot was removed and processed for scintillation counting. The plastic-bound [3H]proline-labeled matrix (i.e. residuum) was solubilized with 0.5 μl NaOH and counted. Percent release of [3H]proline label was calculated and is shown in the figures.

Lipid Peroxidation Determination—Lipid peroxidation in chondrocytes was measured by the cis-parinaric acid (CPA) method described by Hedley and Chow (28). Trypsin-EDTA-released confluent primary chondrocytes were loaded with 10 μM cis-parinaric acid for 1 h at 37 °C and washed. The fluorescence due to parinaric acid was monitored at 37 °C using a luminance spectrometer (LS-5B; Perkin-Elmer, Norwalk, CT) set at 325-nm excitation/405-nm emission.

Preparation of Cell Matrix Extracts—Primary articular chondrocytes in high density (1 × 10⁶/ml) were cultured in 60-mm Petri dishes to confluency, washed three times with HBSS, and set in EBSS, with or without agonist, in a total volume of 1.5 ml for the duration, as indicated in the figure legends. The medium and cell matrix were harvested with a cell scraper in the presence of mixture of inhibitors EDTA (0.5 mM), phenylmethylsulfonyl fluoride (100 μM) and leupeptin (1 μM), and the material was transferred to microcentrifuge tubes. 150 μl of saturated trichloroacetic acid solution was added, and the tubes were incubated for 30 min on ice and microcentrifuged at 12,500 rpm for 10 min. The supernatants were discarded, and the pellets were washed with 50 μl of ethanol, resuspended in 100 μl of sample buffer (Laemmli), and frozen at −70 °C.

SDS-Polyacrylamide Gel Electrophoresis—The samples were thawed and boiled for 5 min with 5 μl of β-mercaptoethanol. They were then cooled on ice, vortexed, spun, and boiled as necessary. A total of 30 μl of each sample was loaded onto a 4% stacking gel and separated in 10% resolving SDS-polyacrylamide gel electrophoresis (PAGE) gel in a mini-PROTEAN II electrophoresis cell (Bio-Rad). Electrophoresis was carried out under the reducing condition of Laemmli (29). Proteins were stained with Coomassie Brilliant Blue.

Protein Detection of Aldehydic Protein Adducts—Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane with Trans-Blot electrophoretic transfer. The blots were incubated with 50 ml of 5% bovine serum albumin (BSA) with Tris-buffered saline (20 mM Tris/500 mM NaCl, pH 7.5) containing 0.1% Tween 20, then washed three times for 15 min with 0.5% BSA with Tris-buffered saline (TBS). For immunodetection, blots were incubated with antibodies diluted in 1% BSA/TBS for 1 h. The mouse monoclonal antibodies MDA2, specific for malondialdehyde-modified lysine, and NAS9, specific for 4-hydroxynonenal-modified lysine, were kindly provided by Dr. W. Palinski, University of California, San Diego (30). The monoclonal antibodies were used at dilutions of 1:2500. The primary antibody was removed, and the blots were washed three times (15 min each) with TBS containing Tween 20. The blots were then incubated in horseradish peroxidase-labeled goat anti-mouse IgG in 1% BSA/TBS (diluted 1:2500) for 1 h at room temperature. Blots were again washed with TBS (15 min each), and proteins were visualized as outlined in the ECL Western blotting protocol (Amersham Pharmacia Biotech). Control blots were also developed, in which incubation with primary antibody was avoided and blots reacted with secondary antibodies and processed as usual. In some cases, when a concentration of monoclonal antibodies, no reactivity to secondary antibody was observed, indicating the immunospecificity of monoclonal antibodies.

Statistical Analysis—Results are expressed as means ± S.E. Total counts in residuum ± S.E. are reported in individual figures or tables. There was a 10% coefficient of variation between the mean and highest and lowest counts in random wells of each experiment. The differences in the means between groups in the same experiment were evaluated by Student’s t test (Statview program), and p < 0.05 was considered statistically significant.

RESULTS

Hydrogen Peroxide and Calcium Ionophore Induced Release of [3H]Proline-labeled Cartilage Matrix (collagen)—The susceptibility of [3H]proline-labeled chondrocyte matrix to oxidant damage was investigated by exposing cultures to a bolus of 2 mM hydrogen peroxide. As shown in Fig. 1, the release of [3H]proline-labeled matrix was significantly enhanced at 4 h in cultures exposed to hydrogen peroxide. The release of labeled matrix by hydrogen peroxide was dose- and time-dependent (data not shown). Hydrogen peroxide is implicated in aggrecan and collagen degradation (19). The data indicate that the release of labeled matrix in the culture media corresponds to the known oxidative-damaging potential of hydrogen peroxide on cartilage matrix.

Chondrocytes were also treated with a variety of agonists (LPS, PMA, fMLP, ConA, and A23187) that have been shown to induce oxidative burst activity in chondrocytes (2–4). Of the various agonists tested, only calcium ionophore A23187 produced a 4- to 10-fold increase in the release of labeled material, as compared with background release by untreated cells (Fig. 1). The A23187 treatment resulted in the release of labeled matrix in dose-dependent manner that was rapid, detected as early as 2 h, reaching a peak by 4–8 h (Fig. 2). It should be noted that in a pulse-chase experiment (Fig. 2), the amount of the labeled material released progressively increased with time and the concentration of agonist, suggesting the release of [3H]proline-labeled material was from mature extracellular matrix (collagen) and not from intracellular [3H]proline-labeled peptides. Also, the soluble release of radioactivity was 90–95% precipitable by trichloroacetic acid (10% trichloroacetic acid not shown), indicating that the activity was associated with peptide. These observations indicate that the calcium ionophore activates chondrocyte-dependent matrix release. Microscopic observation and trypan blue dye exclusion studies ruled out the possibility of chondrocyte lysis as the cause of matrix release by calcium ionophore-treated chondrocytes. The rapid timing of matrix release suggests the involvement of chondrocyte-ROS in matrix degradation.
Antioxidant Enzymes Do Not Inhibit Calcium Ionophore-Induced Release of [3H]Proline-labeled Matrix—To investigate the contribution of chondrocyte-derived ROS in the damage and release of labeled matrix, chondrocyte monolayers were preincubated with either superoxide dismutase (SOD, 100 units/ml), catalase (1000 units/ml), or a combination of both, then treated with or without calcium ionophore A23187 (Fig. 3). Neither SOD, catalase, nor a combination of these two antioxidant enzymes altered the release of matrix, compared with the amount of labeled material released by A23187-activated chondrocytes. These results suggest that neither superoxide anion nor hydrogen peroxide produced by activated chondrocytes are involved in the process of cartilage matrix protein degradation and release. Antioxidant enzymes had no effect on the background amount of labeled matrix released by control unstimulated cells.

Vitamin E Abolishes the Effect of Calcium Ionophore-Induced Release of [3H]Proline-labeled Matrix—Preliminary experiments indicated that vitamin E inhibited the release of labeled matrix in activated chondrocytes. Data from the representative experiment are shown in Table I. Shown in Table I are counts/min of [3H]proline-labeled matrix release at 4 and 8 h. Vitamin E (250 and 500 μM) completely abolished the enhancement of matrix released by calcium ionophore A23187. The abolishing effect of vitamin E was specific and not mediated by diluent ethanol (Table I) on calcium ionophore A23187-treated chondrocytes. Because vitamin E is a chain-breaking antioxidant, the data suggest that the mechanism of matrix release by activated chondrocytes may be mediated by the process of chondrocyte lipid peroxidation (31).

Dose-response Effect of Antioxidant Vitamins E and C on Calcium Ionophore-Induced Release of [3H]Proline-labeled Matrix—The dose-response effect of two natural antioxidant vitamins C and E, on calcium ionophore-induced chondrocyte-dependent matrix release, is shown in Fig. 4. Vitamin C did not show a clear dose-dependent inhibition of [3H]proline matrix release of activated chondrocytes; only at 25 mM was significant vitamin C-induced inhibition seen, as compared with the amount released by A23187-activated chondrocytes (Fig. 4A). Interestingly, vitamin E (100 μM) in some experiments (e.g. Experiment 3) also significantly inhibited the background amount of matrix release by resting control cells, suggesting that vitamin E may be effective in quenching the lipid peroxidative activity of resting chondrocytes.

Measurement of Chondrocyte Lipid Peroxidation Activity—Lipid peroxidation activity was determined in chondrocytes loaded with cis-parinaric acid (10 μM) (28). Serial spectrophotometric readings of resting and activated chondrocytes in the presence of physiological concentration of vitamin E (50 μM) are shown in Fig. 5. There was progressive loss of fluorescence in resting chondrocytes, indicating the basal level of lipid peroxidation activity. Vitamin E inhibited the loss of fluorescence, which suggests that vitamin E modulated the baseline lipid peroxidation activity in chondrocytes. Calcium ionophore

**Fig. 1.** Calcium ionophore- and hydrogen peroxide-induced release of [3H]proline-labeled articular chondrocyte (collagen) matrix. [3H]Proline-labeled monolayer of primary articular chondrocytes in 24-well plates were stimulated with LPS (250 and 100 μg/ml), PMA (1 μg/ml and 100 ng/ml), FMLP (10⁻⁵ and 10⁻⁶ M), calcium ionophore A23187 (2 and 20 μM), and ConA (100 and 10 μg/ml). Cells also were exposed to a bolus of H₂O₂ (2 mM). The 4-h percentage release of labeled matrix (collagen) is shown. The results are means of triplicate sets of wells ± S.E. A representative experiment of three is shown.

**Fig. 2.** Dose- and time-dependent effect of calcium ionophore in a pulse-chase experiment. [3H]Proline-labeled monolayer of primary articular chondrocytes were washed four times with HBSS and incubated in EBSS for 2 h at 37 °C. The cell monolayer were washed again, and experiments were set in the absence or presence of increasing concentrations of calcium ionophore as described under “Materials and Methods.” Shown is the percentage release of labeled matrix at 2 and 4 h.
A23187 (4-bromo-, a nonfluorescent species) caused a rapid and progressive loss of fluorescence; the presence of vitamin E diminished fluorescence loss in A23187-activated chondrocytes. These findings suggest that vitamin E interrupts the lipid peroxidation activity in both resting and activated chondrocytes.

**Inhibitor of Metalloproteases, 1,10-Phenanthroline, Does Not Decrease Calcium Ionophore-induced Release of [3H]Proline-labeled Matrix**—To investigate the possibility that matrix released by activated chondrocytes may be due to the production of metalloproteinases, we determined the effect on matrix release by 1,10-phenanthroline, a general metalloproteinase inhibitor (32). 1,10-Phenanthroline (5–100 μM) did not inhibit the release of matrix by A23187-treated chondrocytes (Fig. 6), suggesting that matrix metalloproteinases are not involved in the process of matrix release by activated chondrocytes. A higher concentration of 1,10-phenanthroline (≥500 μM) resulted in nonspecific release of chondrocyte-matrix monolayer from tissue culture wells. Furthermore, soybean trypsin inhibitor, a serine protease inhibitor, did not decrease calcium-ionophore-induced matrix release (data not shown), indicating that chondrocyte-derived serine proteases are not involved in matrix release.

**Antioxidants and Deferoxamine Inhibit the Release of [3H]Proline-labeled Matrix by Calcium Ionophore-stimulated Articular Chondrocytes**—The effects of the antioxidants, BHT and PG, known to inhibit lipid-free radical reactions, were tested in our model system (33). In addition, the effect of deferoxamine, which has been shown to interrupt the lipid peroxidation process by chelating iron (34), was tested. Both antioxidants and deferoxamine significantly inhibited the release of labeled matrix by activated chondrocytes (Fig. 7), further suggesting that lipid-free radical reaction has a role in matrix damage. Similarly, N-t-butylnaphthylamine (500 μM), a spin-trapping agent, also showed a protective role in inhibiting matrix release by activated chondrocytes (data not shown).

**Immunoblot Analysis of Aldehyde-Protein Adducts in Chondrocyte Matrix Extracts**—Malondialdehyde (MDA) and hydroxynonenal (HNE), major aldehyde products of lipid peroxidation, are believed to be largely responsible for cytopathological effects observed during oxidative stress of lipid peroxidation (35, 36). MDA and HNE react with histidine and lysine residues of proteins to form stable adducts. Demonstration of aldehyde adducts therefore provides clues to the nature of oxidative stress. Protein gel electrophoresis (Fig. 8A) and immunoblot analysis using monoclonal MDA2, specific for MDA-modified lysine (Fig. 8B), and NA59 (30), specific for 4-HNE-modified lysine (Fig. 8C), of chondrocyte extracts are shown in Fig. 8. Compared with control chondrocyte extracts, the extracts from calcium ionophore (5 μM)-treated chondrocytes at 1, 2, and 4 h (lane 4) showed an increased appearance of immunoreactive bands to both MDA2 and NA59, suggesting the formation of activation-dependent adducts. The immunoreactivity of the bands was highest in the 1-h sample and progressively decreased, suggesting a degradative/metabolic process. Together, the data suggest the activation-dependent appearance of MDA- and HNE-protein adducts in chondrocyte extracts, indicating cell-dependent protein oxidation.

We tested the effect of vitamin E on aldehyde-protein adduct formation. Protein gel electrophoresis and immunoblot analysis using NA59 monoclonal antibody are shown in Fig. 9. As shown, NA59 immunoreactivity of the major band increased in calcium ionophore treatment extracts. However, pretreatment and the presence of vitamin E during the activation process resulted in a diminished presence of the major immunoreactivity of the major band increased in calcium ionophore treatment extracts. However, pretreatment and the presence of vitamin E during the activation process resulted in a diminished presence of the major immunoreactivity of the major band increased in calcium ionophore treatment extracts.
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**DISCUSSION**

*In vitro* studies have shown that cartilage matrix components collagen, link-protein, and aggrecan core proteins are degraded by various reactive oxygen species (ROS) and that these processes can be demonstrated to occur in the presence or absence of free-metal ions (14–18). The free-metal ion-independent collagen degradation is a two-step process: oxidation of collagen and subsequent proteolytic cleavage of oxidatively modified collagen (37). The observation that chondrocyte-derived ROS mediate aggrecan degradation (19) and the susceptibility of cartilage matrix proteins to oxidative damage suggest that chondrocyte ROS play a role in cartilage collagen degradation. To decipher the role of chondrocyte ROS in matrix degradation, we developed an *in vitro* model of matrix degradation. The model showed exquisite sensitivity, and the release of 3H-labeled material in the medium corresponded to the potentially damaging oxidative effect of hydrogen peroxide, a known oxidant. Of the various agonists tested to initiate respiratory burst in chondrocytes, only calcium ionophore treatment of chondrocytes, rapidly in a dose-dependent manner, resulted in the release of 3H-labeled material from tissue culture, surface-bound matrix. The release of material was not a result of cell death but was instead mediated by the cell-dependent degradative process on the extracellular matrix (19).

Intervention with antioxidant enzymes, catalase, or superoxide dismutase in the model system did not influence matrix release by calcium ionophore-treated chondrocytes, indicating a lack of a role for chondrocyte-derived hydrogen peroxide or superoxide anion in protein matrix degradation. These observations are contrary to those in the aggrecan degradation model, in which catalase significantly abrogated the release of 35SO4-labeled aggrecan by LPS-stimulated chondrocytes (19).

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![Graph A](image1)  
**FIG. 4.** Dose-response effects of vitamins E (A) and C (B) on the release of [3H]proline-labeled matrix by activated chondrocytes. [3H]Proline-labeled monolayer of primary articular chondrocytes was stimulated with A23187 (10 μM) in the absence or presence of increasing concentrations of vitamin C (0.1–25 mM) and vitamin E (10–250 μM). The results are means of triplicate sets of wells ± S.E. A representative experiment is shown.

![Graph B](image2)  
**FIG. 5.** Measurement of lipid peroxidation in chondrocytes. Chondrocytes were loaded with cis-parinaric acid, 10 μM, for 60 min. Parinaric acid was excited at 325 nm, and fluorescence was collected at 405 nm in 370 °C water-jacketed cuvettes in the presence or absence of vitamin E (50 μM) and 4-bromocalcium ionophore A23187 (5 μM). Shown are serial measurements of a representative experiment.
tion levels of ROS being too inadequate to induce damage or to the spatial arrangement of the collagen matrix in culture, which caused it to be resistant to oxidative damage. Most likely, there are additional explanations for the lack of effect of antioxidant enzymes. Nevertheless, intervention with antioxidant enzymes indicated that chondrocyte ROS plays no role in protein matrix degradation in our model.

The close temporal relationship between chondrocyte-dependent degradation and rapid release of labeled matrix indicated the cause-and-effect relationship of matrix catabolism. As shown, vitamin E significantly inhibited the release of calcium ionophore-induced matrix release. The concentration of vitamin E, which mediated the inhibitory effect, was in micromolar ranges that corresponded to levels found in physiological in vivo conditions. The effect of vitamin E was specific and dose-dependent. In contrast, vitamin C, another physiological antioxidant, showed modest inhibitory effect, and its effect was not dose-dependent. Because vitamin E is a chain-breaking antioxidant, the data suggest that the mechanism of matrix release by activated chondrocytes is mediated by the process of chondrocyte lipid peroxidation (31, 34). Furthermore, lipophilic antioxidants such as BHT and PG, potent inhibitors of lipid-free radical reaction (33), were effective in our model system and support the role of lipid peroxidation in matrix degradation. In addition, we directly measured lipid peroxidation activity in cis-parinaric acid-loaded chondrocytes and showed vigorous constitutive and inducible lipid peroxidation activity in articular chondrocytes (28). The studies also provide evidence that enhancement of lipid peroxidation activity by calcium ionophore preceded the matrix degradation, suggesting there is a causative role in matrix degradation. The injurious effect of lipid peroxidation is initiated by a chain reaction that provides a continuous supply of free radicals, which initiate further peroxidation (31, 34). There is a strong body of evidence supporting the role of lipid peroxidation-linked damage in the cause of cancer, atherosclerosis, aging, and degenerative diseases (34).

Lipid peroxidation involves the process of oxidative decomposition of n-3 and n-6 polyunsaturated fatty acids (PUFA) of membrane phospholipids leading to formation of complex mixtures of lipid hydroperoxides, aldehydic end products such as malondialdehyde and 4-hydroxynonal (35, 36, 38). Bonner et al. (39) documented the lipid profile of human articular cartilage, demonstrating that lipids, especially polyunsaturated fatty acids, accumulate with normal aging of cartilage. Adkisson et al. (40) showed that normal cartilage has low levels of n-6 PUFA and high levels of n-9 fatty acids. The high levels of n-9

FIG. 6. An inhibitor of metalloproteinases, 1,10-phenanthroline, does not inhibit calcium ionophore-induced release of [3H]proline-labeled matrix. [3H]Proline-labeled articular chondrocytes were stimulated with A23187 (10 μM) in the absence or presence of increasing concentration of 1,10-phenanthroline from 5 to 100 μM. The results are means of triplicate sets of wells ± S.E. A representative experiment is shown.

FIG. 7. Antioxidants significantly inhibit calcium ionophore-induced release of [3H]proline-labeled matrix by chondrocytes. [3H]Proline-labeled chondrocyte matrix was stimulated with A23187 (2 μM) in the absence or presence of PG (500 μM), Def (10 μM), and BHT (250 μM). The results are means of triplicate sets of wells ± S.E. A representative experiment is shown.

FIG. 8. SDS-PAGE and subsequent immunoblot analysis of chondrocyte extracts after treatment with and without calcium ionophore A23187 in serum-free EBSS. A, SDS-PAGE; B and C, immunoblots. Primary confluent articular chondrocytes in 60-mm Petri dishes were washed and finally set in serum-free EBSS without (control, lane 1) or with A23187 (5 μM A23187, lanes 2, 3, and 4). Extracts in lanes 2, 3, and 4 were obtained 1, 2, and 4 h, respectively, after culture. Medium-cell matrix was collected as described under “Materials and Methods,” and 30 μl of extract was loaded on SDS-PAGE and transblotted onto nitrocellulose membranes. Subsequently, the membranes were incubated with MDA2 (B) and NA59 (C) monoclonal (1:2500 dilution) for 1 h and processed.
PUFA found in young cartilage are progressively depleted with increasing age and are accompanied by a steady increase in the levels of n-6 PUFA. This trend is especially pronounced in osteoarthritic cartilage (40). Lipid accumulation in chondrocytes also characterizes certain rare osteochondrodysplasias associated with precocious degenerative joint disease (41, 42).

In several models of degenerative arthritis, lipid accumulation generally precedes local tissue degeneration (43, 44). Silberberg and Silberberg (45, 46) have demonstrated an increased incidence of age-dependent osteoarthrosis in C57 inbred mice fed a diet high in saturated fatty acids. Dietary lipids modify the fatty acid composition of cartilage (47). Cartilage tissue from degenerative joints exhibits an accelerated metabolism, an effect that can be reproduced in vitro with normal chondrocytes supplemented with exogenous essential fatty acids (48, 49). Lippiello et al. (50) showed the levels of lipid and arachidonic acid accumulation with an increasing degree of lesion and histological severity in osteoarthrosis.

The identification of aldehydic adducts provides a molecular clue of cell damage mediated by lipid-free radicals. On immunoblot analysis, we identified MDA- and HNE-protein adducts in chondrocyte extracts. The aldehydic adducts were faintly observed in control extracts. On the other hand, a number of specific immunoreactive bands were observed in extracts from activated chondrocytes bands; the 60-kDa band showed maximum reactivity. In a study of serial samples from activated chondrocytes, the intensity of the major band progressively decreased, suggesting metabolic activity. Furthermore, it appears that the pattern of MDA- and HNE-protein adducts was similar in the extracts, suggesting that some proteins may be susceptible to MDA and HNE aldehydic oxidation. Vitamin E in immunoblot study inhibited HNE adduct formation. In vitro, vitamin E reverses the toxic oxidative effect of linolenic acid-impaired chondrocyte cell function and protects against cellular peroxidation in cartilage (48). In the Framingham Knee Osteoarthritis Cohort Study (51), the population having medium to higher intake of vitamin C, beta carotene, and vitamin E had a reduced risk of progression of knee osteoarthritis as assessed using radiography.

The unique distribution of lipids in cartilage, which changes significantly with age, and dietary intake of lipids could influence the lipid peroxidizability of cartilage (52). It is possible that alteration in lipid metabolism and oxidative stress could be a catalyst for cartilage aging. In chondrocytes, reactive radicals induced by NADPH-oxidase may initiate lipid peroxidation. In particular, superoxide radicals could combine with other naturally occurring radicals, such as nitric oxide, resulting in the formation of peroxynitrite, a powerful oxidant known to initiate lipid peroxidation. Chondrocytes have been shown to produce nitric oxide constitutively and in an activation-dependent manner (53). Collectively, chondrocyte lipid peroxidation appears to play both a physiological and a pathological role in cartilage. Age-related changes in the lipid composition of cartilage could push the normally contained lipid peroxidation process into a state of uncontrolled oxidative stress, leading to the oxidation of cartilage collagen. Oxidation of collagen could cause fragmentation, which alters the material properties of collagen fibrils, thereby making them more brittle and prone to mechanical fatigue failure. Such failure could initiate osteoarthrosis.
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