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Malondialdehyde oxidation of cartilage collagen by chondrocytes

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Summary

Objective: The damage to cartilage collagen is a central event in the pathogenesis of cartilage aging and osteoarthritis (OA). We have previously developed an *in vitro* model of cartilage degradation which shows that chondrocyte-dependent lipid peroxidation mediates cartilage collagen degradation. The goal of our study was to investigate the role of vitamin C in this degradation model and to investigate effect of chondrocyte-dependent lipid peroxidation in the oxidation of cartilage collagen.

Methods: We studied primary articular chondrocytes. Effect of vitamin C was investigated in the previously described model. Serum-free stimulated and unstimulated chondrocyte-matrix extracts were subjected to SDS-PAGE and immunoblot analysis. Malondialdehyde (MDA)-protein oxidation of cartilage proteins was demonstrated by the reactivity of chondrocyte extracts to a monoclonal antibody, MDA2, which detects MDA–lysine adducts.

Results: Vitamin C treatment of chondrocyte cultures resulted in significant enhanced incorporation of 3H-proline label in cell-matrix. Cells treated with vitamin C, as compared to control untreated cells showed decreased spontaneous release of labeled matrix. Vitamin C treated or not treated chondrocytes responded comparably to stimulation with the agonist calcium ionophore A23187. The serum-free *in vitro* culture of chondrocytes resulted in MDA-protein oxidation. The treatment of chondrocytes with A23187 resulted in the enhancement of MDA-protein oxidation. The immunoblot reactivity pattern of extracts to MDA2 antibody and to polyclonal anti-type II collagen antibody was somewhat similar, which suggests that these two different types of antisera exibit a crossreaction to chondrocyte proteins. Chondrocyte extracts were pretreated both with and without pure collagenase, and then subjected to immunoblot analysis. Only collagenase treated extracts showed a disappearence, or significant reduction, of larger than 60 kDa size MDA2 immunoreactive proteins. This suggests that the proteins that disappeared after the enzyme treatment were collagen proteins and which had also been modified by MDA oxidation.

Conclusions: These observations suggest that collagen hydroxylation of matrix by vitamin C does not play a role in this model of chondrocyte-dependent collagen degradation. Also, this study demonstrates that chondrocyte-derived lipid peroxidation product MDA mediates oxidation of cartilage collagens. Oxidative modification of cartilage collagen *in vivo* could result in alteration of biochemical and biophysical properties of cartilage collagen fibrils, making them prone to degradation, thus initiating the changes observed in aging and OA.

Key words: Chondrocytes, Cartilage, Lipid peroxidation, Vitamin C, Matrix degradation, Collagen, Malondialdehyde, Protein oxidation.

Introduction

Damage to cartilage collagen is a central event in the pathogenesis of cartilage aging and osteoarthritis (OA)[1–5]. There is a strong association between the progressive reduction in content of collagen and its tensile properties during aging and OA[2]. In both aging and OA, the damage to collagen originates around chondrocytes and extends into the cartilage, with progressive degeneration[3]. Furthermore, in chondrodystrophies, abnormalities of collagen types II, IX, and XI are associated with a predisposition to development of OA[4]; transgenic animals with defective collagen gene are also predisposed to degenerative arthritis[5].

Collagen is the most abundant protein in the body, and the *in vivo* turnover of mature collagen is extremely slow[6]. The tensile strength of cartilage collagen is enhanced by intra- and inter-fibrillar cross-linking of collagen; the major cross-linking residue in mature type II collagen fibril is pyridinoline[7]. Nonenzymatic glycosylation or glycation of collagen is influenced by changes in the microenvironment. For example, advanced glycation end (AGE) products accumulate according to the severity of diabetic complications[8]. The processes of glycation and oxidation, often called glycoxidation, delineate the formation of a novel class of Maillard products resulting from the oxidative cleavage of Amadori products that are characterized by pentoside cross-links[9]. Furthermore, the formation of AGE products is accelerated by metal-catalyzed oxidation[10]. AGE-modified proteins stimulate a variety of cellular responses via specific cell surface receptors[11,12]. Glycated collagen has been shown to promote oxidation of polyunsaturated fatty acids[13].

Several studies have established the age-related insolubility of collagen as well as an increase in pentosidine (but not pyridinoline cross-links) in aged versus young

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cartilage[14,15]. Furthermore, pentosidine cross-links do not increase in experimental OA[16]. Cartilage changes its color with age from a white appearance to yellowish brown with accumulation of fluorescent pigment, thought to be lipid–protein adducts[14–16].

Cartilage exhibits a unique lipid profile[17]. Chondrocytes express lipid peroxidation activity; the antioxidant, vitamin E plays an important role in growth and bone development[17–22]. In a previous study, we have shown that normal articular chondrocytes express constitutive levels of lipid peroxidation activity, which is enhanced by treatment with agonists such as calcium ionophore A23187[23]. Additionally, in an *in vitro* arthritis model, we demonstrated that the enhancement of chondrocyte lipid peroxidation mediates cartilage collagen degradation[23].

Vitamin C is well documented to cause collagen hydroxylation by modification of prolyl and lysl residues[24,25]. Connective tissue cells including chondrocytes cultured in absence of vitamin C result in collagen underhydroxylation with impaired collagen secretion[24]. Ascorbate treated cells produce demonstrably more matrix than cells cultured in the absence of the vitamin. In previous description of our model of collagen degradation we did not culture primary chondrocytes with vitamin C[23]. It is therefore possible because in absence of vitamin C, collagen underhydroxylation and unstable triple-helical collagen could have created experimental conditions in which matrix was sensitive target for chondrocyte-dependent degradation. Furthermore lack of antioxidant effect of vitamin C could also have influenced chondrocytes in a manner to cause collagen degradation. To test these possibilities we investigated the role of vitamin C in our model.

Malondialdehyde (MDA) and hydroxynonenal (HNE), are major aldehyde products of lipid peroxidation, believed to be responsible for cytopathological effects observed during lipid peroxidation induced oxidative stress[26,27]. MDA and HNE react with the histidine and lysine residue of proteins to form stable adducts. The demonstration of aldehydic adducts *in vivo* or *in vitro* provides clues into the nature of oxidative stress. The second goal of this study was to characterize the formation of MDA–aldehydic adducts in the control and stimulated chondrocyte-matrix extracts. Importantly, our aim was to demonstrate that cartilage collagen is subjected to aldehydic oxidation.

Materials and methods

REAGENTS

Lipopolysaccharide (LPS) from Escherischia coli 0127:B8, calcium inonphore A23187, high-purity type VII collagenase, and all chemical reagents were purchased from the Sigma Chemical Company (St. Louis, MO). Dulbecco's minimum essential medium (DMEM), fetal bovine serum (FBS), Hank's balanced salt solution (HBSS), Earl's balanced salt solution (EBSS), L-glutamine, gentamicin, HEPES buffer, penicillin, and streptomycin were purchased from GIBCO (Grand Island, NY).

ISOLATION OF RABBIT ARTICULAR CHONDROCYTES

NZW rabbits [6–8 pounds (2.7–3.6 kg)] of either sex were killed by i.v. injection of Beuthanasia-D special (Schering Corporation, Kenilworth, NJ). Chondrocytes were isolated as described previously[28]. The viability of chondrocytes was confirmed by trypan blue exclusion method. Primary chondrocytes were resuspended in 10% FBS in DMEM containing antibiotics (1%) and HEPES buffer (10 mM, pH 7.4) (complete media).

EXPERIMENTAL DESIGN

Primary rabbit articular chondrocytes were distributed into 24-well plates at a concentration of $1–2\times10^5$ 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 multiwell plates were labeled with 1–2.5 μCi/well with 3H-proline during the last 24 h of cell culture. The cell monolayer was washed at least four to five times with warm HBSS by flipping the plates to remove unincorporated proline from the matrix. Albumin or serum-free EBSS was added to 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% CO_2 incubator for 4–24 h. One hundred microliter aliquot was removed and processed for scintillation counting. The plastic-bound 3H-proline labeled matrix (i.e., residuum) was solubilized with 0.5 M NaOH and counted. Percent release of 3H-proline labeled was calculated and is shown in Fig. 1.

PREPARATION OF CELL-MATRIX EXTRACTS

Primary articular chondrocytes in high density (1×10^6/ml) were cultured to confluency in 60 mm petri dishes, washed three times with HBSS, and set in EBSS with or without agonists (calcium ionophore, A23187 or LPS) in a total volume of 1.5 ml and cultured for the duration, as indicated in the figure legends. Both medium and cell-matrix were harvested together, using a cell-scraper in the presence of a cocktail of inhibitors [ethylenediaminetetraacetate (EDTA) (0.5 M), phenylmethylsulfonyl fluoride (PMSF) (100 μM), leupeptin (1 μM)] and the material transferred to microcentrifuge tubes. One hundred and fifty microliters of saturated trichloroacetic acid solution was added, and tubes were incubated for 30 min on ice and centrifuged at 12 500×rpm for 10 min. The supernatants were discarded, and pellets were washed with 50 μl of ethanol and suspended in 100 μl of Laemmli buffer[27]; samples were frozen at −70°C.

PROTEIN GEL ELECTROPHORESIS

The frozen samples were thawed and boiled for 5 min with 5 μl of β-mercaptoethanol. The samples were cooled on ice, vortexed, spun, and boiled. A total of 30 μl of each sample was loaded into a 4% stacking gel and separated in 10% resolving SDS-PAGE gel in a mini-PROTEAN II electrophoresis cell (Bio-Rad). Electrophoresis was carried out under reducing condition of Laemmli[29]. Protein gels were stained with Coomassie Brilliant Blue.

IMMUNODETECTION OF ALDEHYDE–PROTEIN ADDUCTS

Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane using a Trans Blot electrophoretic transfer cell unit. The blots were incubated with 50 ml of 5% BSA in Tris-buffered-saline (TBS: 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 TBS. For immunodetection, the blots were incubated overnight with the antibodies. The mouse monoclonal antibodies MDA2,

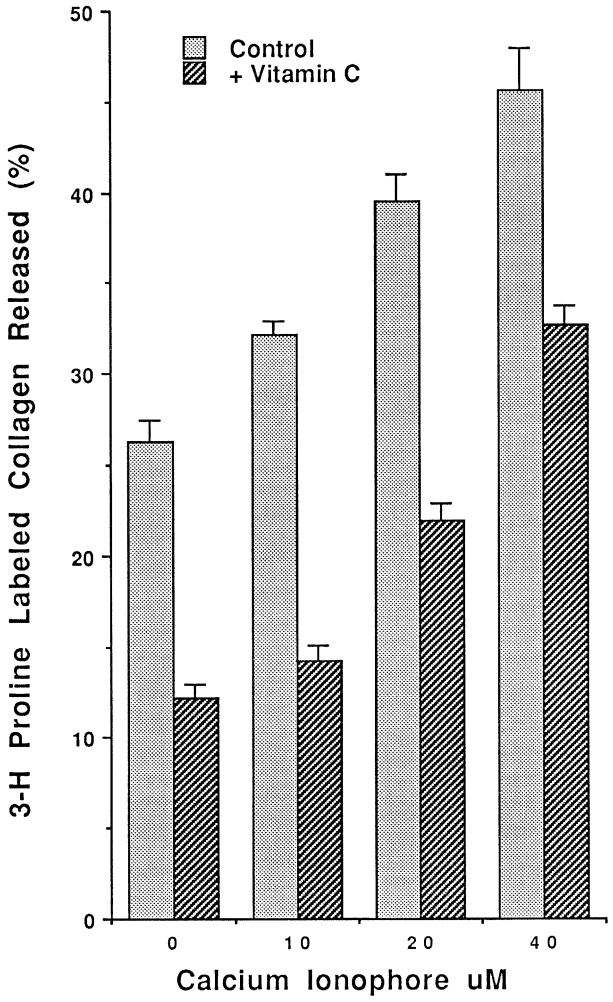

Fig. 1. Effect of vitamin C in collagen degradation model. Monolayer of primary articular chondrocytes in 24-well plates were conditioned with 5 µg/ml of vitamin C for 24 h and medium exchanged with fresh complete medium with 100 µg/ml of vitamin C. Control cultures were identically treated without vitamin. The cells were cultured for 48 h and labeled with 3H-proline. Assays were initiated in serum-free EBSS in the presence and absence of increasing concentration of calcium ionophore as shown. The 4-h percentage release of labeled collagen is shown. The results are means of triplicate sets of wells±S.E. A representative experiment of six is shown.

specific for MDA-modified lysine was provided by Dr W. Palinski, University of California, San Diego[30]. The mAbs were used at a dilution of 1:2500. Polyclonal mouse anti-type II collagen antibodies, which recognizes native and degraded type II collagen fragments, were provided by Drs D.D. Brand and J.M. Stuart from the Veterans Affairs Medical Center, Memphis, TN[31]. These antibodies were supplied at 500 ng/ml in 2% normal goat serum in PBS. The polyclonal antibodies were used at a dilution of 1:1000. The primary antibody was removed after overnight incubation, 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 the proteins were visualized as outlined in the ECL Western blotting protocols (Amersham). Immunoblots were also developed in incubation buffer from which primary antibodies were omitted, then reacted with secondary antibodies and processed as usual. No reactivity to monoclonal or polyclonal antibodies was observed in control blots indicating immunospecificity of mAbs and polyclonal antibodies (data not shown).

COLLAGENASE DIGESTION OF CARTILAGE EXTRACTS

Pellets of chondrocyte-matrix extracts in 15 µl reaction volume containing 50 mM Tris–HCL, pH 7.2, 10 mM $CaCl_2$ and 2 U high-purity type VII collagenase were vortexed and digested for 1 h at 37°C. As controls, undigested cell-matrix extract was dissolved in the reaction buffer lacking collagenase, incubated for 1 h at 37°C or kept in an ice slurry. One hundred microliters of sample buffer and 5 µl of β-mercaptoethanol was added to each tube and boiled. About 35 µl of each sample was subjected to protein and immunoblot analysis.

Results

EFFECT OF VITAMIN C ON COLLAGEN DEGRADATION MODEL

Confluent monolayers of primary articular chondrocytes were exposed to various concentrations of vitamin C (25–100 µg/ml) which resulted in morphological changes in cell-monolayer. The cells appeared rounded and were easily detachable. Vitamin C is reported to induce apoptosis in chondrocytes[32]. To avoid chondrocyte apoptosis cell monolayers were conditioned with 5 µg/ml of vitamin C for 24 h, which did not result in visible changes in cells, and then medium changes to complete medium with 100 µg/ml of vitamin C. Control cells were identically processed but without vitamin C. The cells were further cultured for 24–48 h and labeled with 3H-proline. Collagen degradation assays were initiated as described above. It should be noted that vitamin C was not present during the assay period.

Treatment of chondrocyte cultures in presence of vitamin C resulted in enhanced incorporation of 3H-proline in cell-matrix as compared to cell-matrix not treated with vitamin. Enhanced incorporation exceeded by 15–100% as compared to control cultures (data not shown). During assay conditions vitamin C treated cell-matrix consistently showed decreased spontaneous release of labeled collagen as compared to control cell-matrix. The net effect of decreased spontaneous release of labeled collagen and enhanced amounts of total collagen matrix in vitamin treated cells resulted in significant reduction in calculated percent release of labeled collagen as compared to percent collagen release in control untreated cells. On the other hand, both vitamin C treated and control cells showed comparable enhancement of collagen degradation upon exposure to increasing concentration of agonist calcium ionophore A23187, as shown in Fig. 1. These observations suggest that vitamin C treatment of chondrocyte cultures do not influence collagen degradation in response to chondrocyte activation with A23187.

MDA–PROTEIN ADDUCTS IN CHONDROCYTE EXTRACTS

Chondrocyte-matrix extracts, treated with or without calcium ionphore A23187 for 4 h, were subjected to SDS-PAGE, and the blots were subsequently probed with

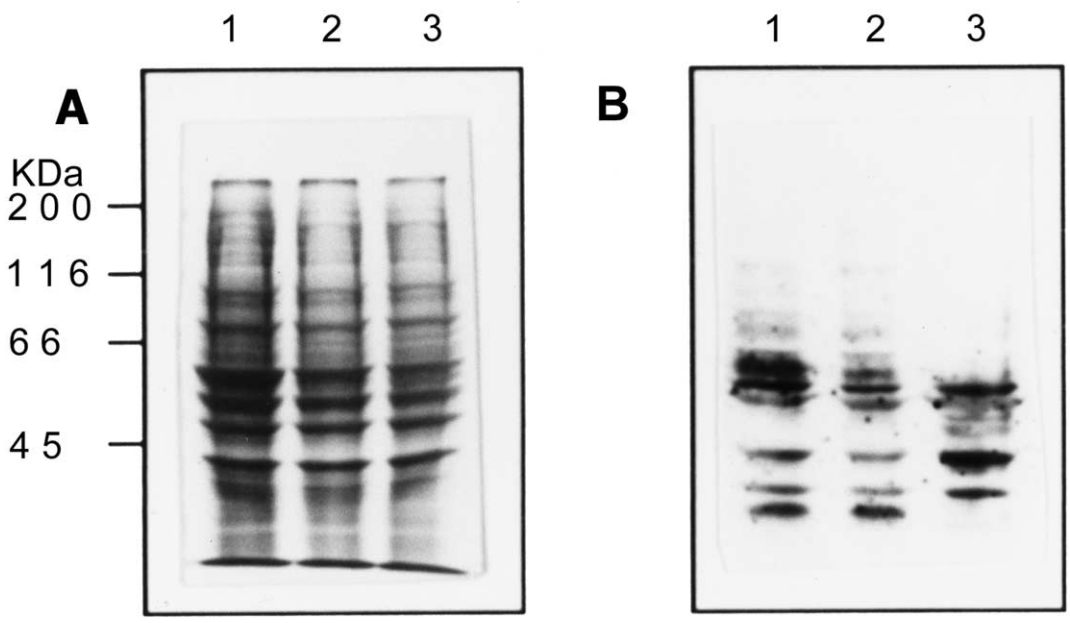

Fig. 2. SDS-PAGE and subsequent immunoblot analysis of chondrocyte extracts. (A) SDS-PAGE. (B) Immunoblot. Primary confluent articular chondrocytes in 60 mm petri dishes were washed and finally set in serum-free EBSS without (control, lane 1) or with A23187 (5 µM, lane 2) and A23187 (50 µM, lane 3) in a final volume of 1.5 ml. Four-hour extracts of medium cell-matrix were collected, as described in the Section Methods and 30 µl of extract was loaded on SDS-PAGE and transblotted to nitrocellulose membrane. Subsequently, the membrane was incubated with MDA2 monoclonal (1:2500 dilution) overnight and processed.

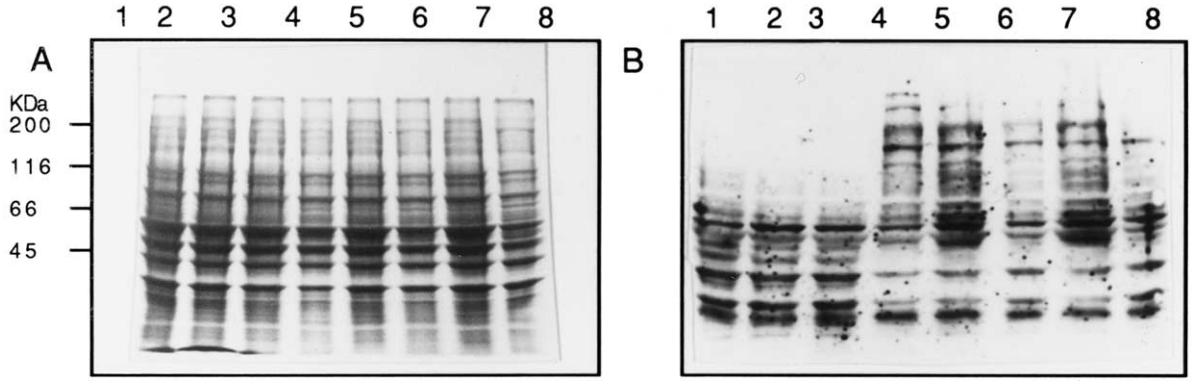

Fig. 3. SDS-PAGE and subsequent immunoblot analysis of chondrocyte extracts. (A) SDS-PAGE. (B) Immunoblot. Protein and immunoblot analysis of timed samples of control (lanes 1, 3, 5, and 7) and A23187 (5 µM) stimulated (lanes 2, 4, 6, and 8) extracts. Lanes 1 and 2, 3 and 4, 5 and 6, and 7 and 8 were extracted at 0, 2, 4, and 8 h after incubation, respectively. Extracts of medium-cell matrix were collected as described, and 30 µl of extract was loaded on SDS-PAGE and transblotted to nitrocellulose membrane. Subsequently, the membrane was incubated with MDA2 monoclonal (1:2500 dilution) overnight and processed.

monoclonal antibody MDA2. As shown in Fig. 2, the overall intensity of immunoreactive bands decreased in the A23187-stimulated cell extracts, as compared to the untreated cell extracts. Noticeably, immunoreactive bands ≥60 kDa disappeared particularly in 50 µM A23187-stimulated extracts. At the same time, the density of the lower molecular weight MDA2 reactive bands increased in the stimulated extracts. In contrast, there was no significant difference in the intensity of the Coomassie Brilliant Blue stained protein bands between unstimulated and stimulated extracts (Fig. 2A). These observations highlight the presence of MDA-protein reactive products in serum-free *in vitro* cultured chondrocyte extracts. Stimulation of chondrocytes with calcium ionophore results in the formation and disappearance of MDA-oxidized proteins.

To further investigate the dynamics of MDA-adduct formation, the chondrocytes were stimulated both with or without 5 µM calcium ionophore A23187 for various periods (0, 2, 4, and 8 h). In Fig. 3, both control (lane 1) and A23187-substituted extract (lane 2) showed identical MDA2 immunoreactive pattern at 0 h, suggesting that the addition of calcium ionophore did not influence MDA2 immunoreactivity. A similar pattern of immunoreactivity was observed in control 2-h extracts (lane 3). In contrast, the 2-h A23187-stimulated extract (lane 4) exhibited the appearance of new immunoreactive bands of >60 kDa. A

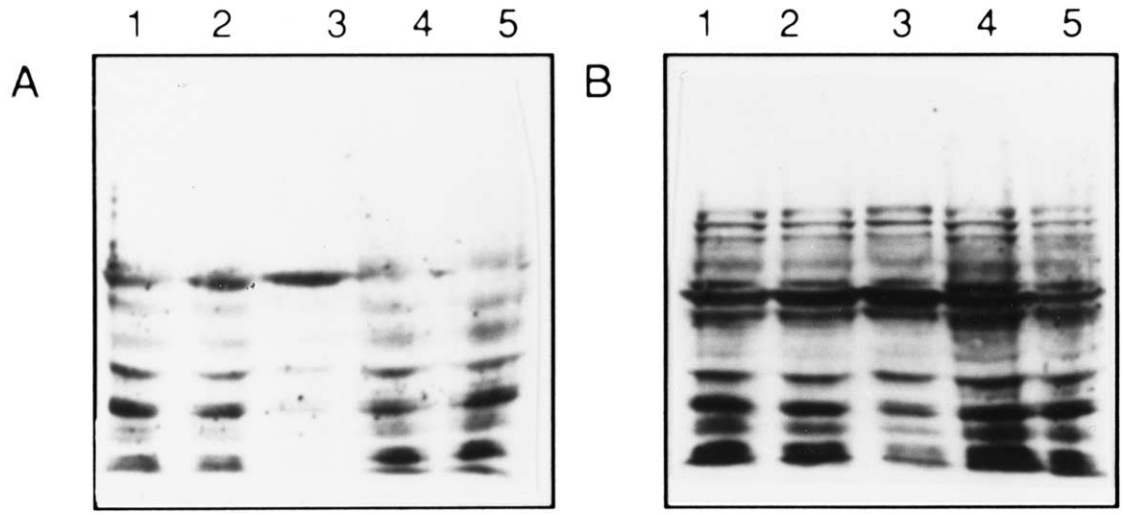

Fig. 4. Immunoblot analysis of chondrocyte extracts. (A) MDA2 reactive immunoblot. (B) Polyclonal mouse anti-type II collagen antibody reactive immunoblot. Primary confluent articular chondrocytes in 60 mm petri dishes were washed and set in serum-free EBSS. Control chondrocytes were untreated (lane 1). Chondrocytes in lanes 2 and 3 were stimulated with 40 and 400 μg/ml of LPS, respectively. Chondrocytes in lanes 4 and 5 were stimulated with 4 and 40 μM of calcium ionophore A23187. All the extracts were collected after 4 h of cell culture and 30 μl of extracts was loaded on two duplicate SDS-PAGE gels, and both were transblotted to nitrocellulose membrane. Subsequently, membrane in A was incubated with MDA2 monoclonal (1:2500 dilution), and membrane B was incubated with polyclonal mouse anti-type II collagen antibodies (1:1000 dilution) overnight and processed.

number of these newly appearing MDA2-reactive bands progressively decreased in the 4- and 8-h extracts (lanes 6 and 8). In addition, some immunoreactive bands either decreased in intensity, or disappeared altogether. The appearance and disappearance of new MDA2-reactive bands was also observed in the control extracts (lanes 5 and 7); however, MDA2 reactive bands appeared after 4–8 h in the control extracts, while in the stimulated extracts these bands appeared within 2 h. The distribution or intensity of Coomassie Brilliant Blue stained protein bands between the stimulated and unstimulated extracts did not show any significant change. These data represent the kinetics of presence of MDA-protein oxidation products in unstimulated and stimulated serum-free chondrocyte extracts, which indicates activation-dependant acceleration of the MDA oxidative process in chondrocytes.

PROTEIN OXIDATION OF CARTILAGE COLLAGEN(S)

Next, we investigated whether collagen, the key extracellular matrix protein in cartilage, is subjected to MDA-protein oxidation. As shown in Fig. 4A, treatment with a higher (400 μg/ml) concentration of LPS resulted in the disappearance of the ≤60 kDa MDA2 immunoreactive bands, as compared to control or 40 μg/ml LPS-treated extracts. Calcium ionophore A23187-treated extracts showed diminished MDA2 immunoreactivity of ≈60 kDa bands and increased density of ≤45 kDa bands. The pattern of immunoreactivity to anti-type II collagen antibodies somewhat closely resembled the pattern of MDA2-reactive bands (Fig. 4B). However, additional high molecular weight bands (100 kDa) observed by polyclonal antisera may represent mature collagen molecules, while low molecular weight bands are probably collagen fragments. In addition, it should be noted that the pattern of immunoreactivity to anti-type II collagen antibodies in the control and stimulated extracts was almost the same or similar. Taken together, the similarity of immunoreactivity patterns by simultaneous probing with MDA2 and anti-type II collagen antibodies, the data suggests that some of the proteins reacting to MDA2 are most likely collagen molecules.

Following that, we used the specificity of high-purity collagenase digestion as a tool to discriminate between the presence of collagenous and noncollagenous proteins in chondrocyte extracts. As shown in Fig. 5B, the MDA2 immunoreactivity pattern observed, in the control and stimulated extracts, incubated at 4 and 37°C, respectively, was similar. It should be noted that incubation at 37°C did not result in the disappearance of proteins or immunoreactive bands, as compared to the incubation at 4°C, which suggests that the extracts did not contain functional enzymes. If proteolytic enzymes were present in extracts incubation at 37°C it would have resulted in spontaneous proteins degradation and consequently resulted into alteration of the stained protein and immunoreactive bands. In addition, there are more protein bands in lanes 5 and 6, which we attribute to the addition of enzyme collagenase. Furthermore, to the naked eye there are no discernable changes in Coomasie Brilliant Blue stained protein bands between extracts with or without collagenase. On the other hand, only exogenous addition and treatment with pure collagenase resulted in the disappearance of high molecular size bands, as well as the significant reduction in the density of main MDA2 immunoreactive bands. This indicates that the protein bands that disappeared were true collagens and that the same proteins had also been modified by MDA-protein oxidation, as shown by their reactivity to MDA2 antibodies.

Discussion

Cartilage is specialized resilient tissue, which may be prone to early aging due to avascularity, hypoxic condition,

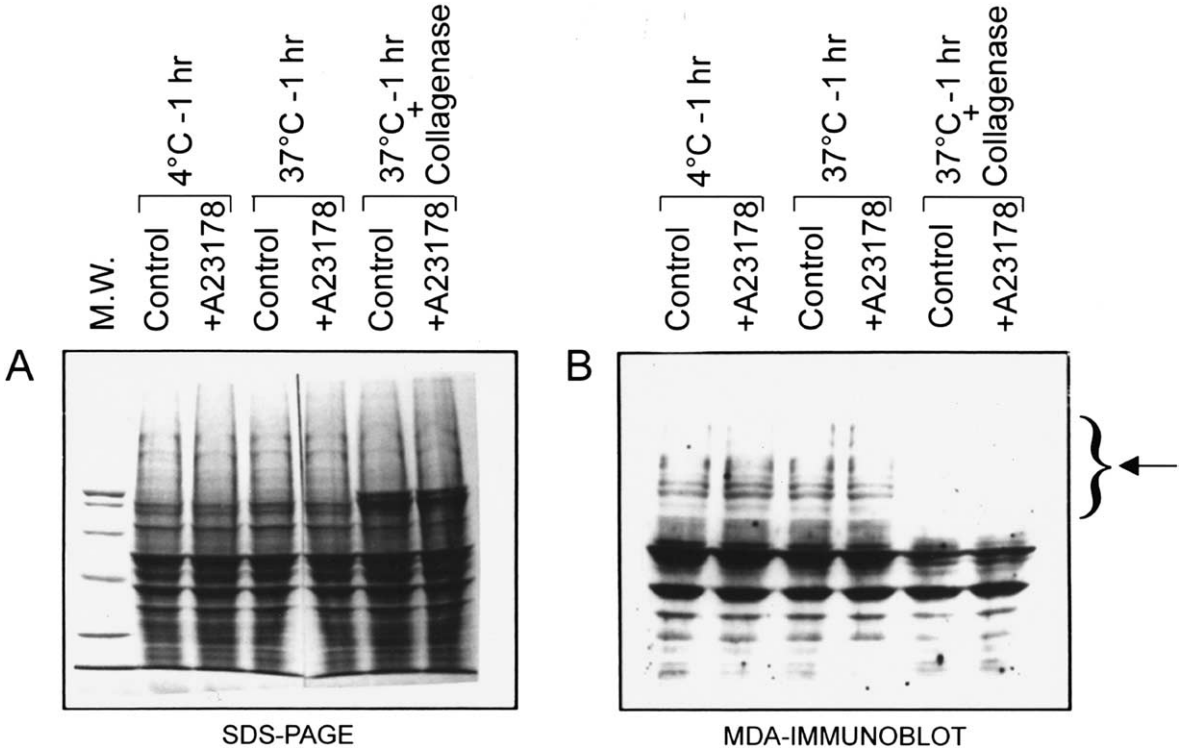

Fig. 5. SDS-PAGE and subsequent immunoblot analysis of chondrocyte extracts. (A) SDS-PAGE. (B) Immunoblot. All the extracts from control unstimulated and 5 µM A23187 stimulated primary articular chondrocytes were obtained after 2 h of culture. Equal volumes of pooled control (lanes 1, 3, and 5) and stimulated extract (lanes 2, 4, and 6) were distributed into triplicate sets of microtubes, precipitated with TCA and pellets washed with ETOH, and dry pellets dissolved in 15 µl of 50 mM Tris–HCL, pH 7.2. 10 mM $CaCl_2$ buffer without (lanes 1, 2, 3, and 4) or with 2 U collagenase (lanes 5 and 6). The extracts were incubated at 4°C (lanes 1 and 2), at 37°C (lanes 3 and 4) and with collagenase 2 units (lanes 5 and 6) for 1 h, then processed as described in the Section Methods. Thirty-five microliter of extract was loaded on SDS-PAGE and transblotted to a nitrocellulose membrane. Subsequently, the membrane was incubated with MDA2 monoclonal (1:2500 dilution) overnight and processed. Arrow indicates MDA2 reactive protein bands which disappeared following collagenase enzyme treatment.

or subjection to repetitive compression, which leads to cycles of ischemia and reperfusion[1]. The precise mechanism of collagen abnormality induced in aging or OA is not known[3]. Collagen is normally resistant to enzymatic digestion[33]. However, there is a unique class of zinc-dependent enzymes, matrix metalloproteinases, which selectively degrade collagen[34]. A large body of evidence highlights the important role of matrix metalloproteinases in OA[35,36].

In addition to the enzymatic mechanism of collagen degradation, collagen is susceptible to oxidative damage[37,38]. *In vitro* studies have shown that collagen can be degraded by various reactive oxygen species. This process was demonstrated in both the presence and absence of metal-free-ions[39]. The metal-free-ion independent mechanism of collagen degradation is a two-step process: first the oxidation of collagen, and secondly the proteolytic cleavage of oxidatively modified collagen[39]. Collagen is characterized by a triple-helical structure composed of repeat sequences of Gly–X–Y, where X and Y is often represented by proline and hydroxyproline[33]. In a model proline peptide for collagen, oxidative damage resulted in proline oxidation that led to fragmentation, which was accompanied by the formation of 2-pyrrolidone cross-links[40].

Our recent observations, suggests that chondrocytes exhibit constitutive and inducible lipid peroxidation activity[20]. Furthermore, we showed the importance of chondrocyte-derived lipid peroxidation in cartilage matrix protein (collagen) degradation[23]. Since the above published report, a question has risen as to the role of vitamin C in our model of collagen degradation. Present investigations on these lines confirm the presence of demonstrably more collagen matrix in chondrocytes cultured in the presence of vitamin C than those cultured in the absence of vitamin. Furthermore, it appeared that collagen matrix derived in vitamin C treated chondrocytes was more stable as indicated by diminished spontaneous release of labeled collagen. Most likely this may be attributed to collagen hydroxylation and stable secreted triple-helical collagen[24,25]. In contrast, vitamin C treatment of chondrocytes did not influence reactivity to lipid oxidative stress induced by calcium ionophore. Ascorbate conditioned cell-matrix as well control cell-matrix were subjected to degradation upon chondrocyte stimulation suggesting that lipid oxidative stress equally causes degradation of underhydroxylated and hydroxylated collagen molecules. Collectively, these observations suggest that vitamin C treatment of chondrocyte culture does not significantly influence our model of collagen degradation. It should be pointed out that vitamin C as a physiological antioxidant showed modest inhibitory effect during degradation assay and its effect was not dose-dependent as was reported previously[23].

There is a large body of evidence which indicate the tissue-damaging role of lipid peroxidation[27]. Damage linked to lipid peroxidation has been implicated as a cause of cancer, atherosclerosis, aging, and degenerative diseases.

The injurious effect of lipid peroxidation is initiated by a chain reaction that provides a continuous supply of free radicals that initiate further peroxidation[27]. Lipid hydroperoxide and its products, MDA and 4-HNE, are major toxic aldehydic products that mediate oxidative damage[26,27]. Due to their increased polarity and long half-lives, as compared to reactive oxygen species, the lipid free radicals may be able to migrate from its point of production, within the immediate vicinity of the cells, to extracellular tissue sites. There these small-reactive molecules could permanently modify extracellular matrix proteins. Detection of aldehyde modified adducts provide molecular imprints of lipid peroxidation induced protein oxidation, and therefore infer its role in the disease pathogenesis[9,26,27].

Using the monoclonal antibody MDA2, we were able to identify specifically MDA–lysine adducts in chondrocyte extracts. Interestingly, malondialdehydic adducts were also present in 0-h extracts of primary *in vitro* cultured articular chondrocytes, which suggests that chondrocytes constitutively and continuously produce these aldehyde–protein adducts. It is possible that *in vitro* exposure to ambient concentrations of oxygen (high concentration), in contrast to the *in vivo* hypoxic microenvironment of cartilage, could cause oxidative stress in chondrocytes, which would result in the sustained *in vitro* formation of aldehydic adducts. Using a fluorescence method of monitoring lipid peroxidation in living chondrocytes (*cis*-parinaric acid loaded chondrocytes), employed in our previous study, we observed progressive loss of fluorescence in resting chondrocytes, which indicated a basal level of lipid peroxidation activity[23]. Furthermore, we demonstrated the enhancement of a baseline level of lipid peroxidation upon treatment with agonists, such as calcium ionophore A23187, which indicates an activation-associated enhancement of chondrocyte lipid peroxidation[23]. In our present study, the immunoblot analysis of the control and stimulated extracts collected at various time-points, suggests that the newly formed MDA adducts were ≥60 kDa in size. These MDA-modified proteins of high molecular weight disappeared in the extracts, which also suggests rapid metabolism of oxidatively modified proteins.

The biochemical and structural integrity of collagens is critical to the preservation of cartilage; the alteration of biochemical and biophysical properties of collagen could herald cartilage degradation[36]. We therefore surmised that it was important to explore whether cartilage collagen is subjected to aldehydic protein oxidation. To decipher the role for lipid aldehyde oxidation of collagen, we employed simultaneous probing with monoclonal MDA2 and polyclonal anti-type II collagen antibodies, with the latter recognizing both native and damaged collagen fragments. Using these two antisera, the pattern of proteins identified in chondrocyte extracts were somewhat similar, which indicates that there was considerable crossreactivity between anti-type II collagen and MDA2 antisera.

Additionally, polyclonal anti-type II collagen antibodies identified high molecular weight proteins, which were probably mature collagen molecules. We confirmed the collagenous nature of the proteins in the extracts through a specific collagenase treatment. Only collagenase treatments resulted in loss and diminution of MDA-reactive bands larger than 60 kda size, which indicates that the disappeared protein bands were true collagens, because these proteins were susceptible to specific collagenase digestion. The data demonstrate that collagenase susceptible protein bands were also modified by MDA, as suggested by their reactivity to MDA2 antibodies. Taken together, these observations highlight the existence and formation of the malondialdehydic oxidation of cartilage collagen by chondrocytes.

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