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Inflammation Research

Hypoxia-induced hyaluronan synthesis by articular chondrocytes: the role of nitric oxide

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Abstract. *Objective:* Articular cartilage is an avascular tissue in which chondrocytes are exposed to hypoxic conditions. We previously demonstrated that reactive oxygen species (ROS) induced apoptosis of chondrocytes. We also demonstrated that nitric oxide (NO) was induced when chondrocytes were exposed to hypoxia and that NO inhibited the ROS-induced apoptosis. Hyaluronan (HA) is a high molecular weight glycosaminoglycan whose antioxidative effects have been reported. The purpose of the present study was to determine whether HA synthesis was induced in chondrocytes exposed to hypoxia, and, if so, whether the hypoxia-induced HA synthesis is regulated by NO.
Methods: Bovine articular chondrocytes were used in this study. Levels of HA were determined by the sandwich enzyme-binding assay. Expression of HA synthase (HAS) was determined with reverse transcription-polymerase chain reaction. The production of NO was examined using the Griess reaction. We also determined inducible nitric oxide synthase (iNOS) enzyme synthesis using the histochemistry and Western blot analysis.
Results: Chondrocytes cultured under hypoxic conditions exhibited enhanced HA synthesis. When the NO inhibitors, L-NMMA and L-NAME, were added, the hypoxia-enhanced HA levels in the culture medium were significantly inhibited.
Conclusions: Endogenous NO synthesis plays an important role in hypoxia-enhanced HA synthesis.

Key words: Chondrocyte – Hyaluronan – Hypoxia – Nitric oxide

Introduction

Articular cartilage is an avascular tissue that derives its nutrition and oxygen supply by diffusion from the synovial fluid and the subchondral bone. Chondrocytes at the articular surface are estimated to be exposed to approximately 6 to 10 % O_2, whereas chondrocytes in the deepest layers of the articular cartilage may have access to 1 to 6 % O_2, or even less [1, 2]. Therefore, chondrocytes normally display a metabolism adapted to anaerobic conditions. However, in pathological conditions like osteoarthritis and rheumatoid arthritis, the oxygen tension in synovial fluid is subject to fluctuations as a consequence of the ischemia–reperfusion phenomenon [3].

Previously, we hypothesized that chondrocytes were susceptible to the attack of reactive oxygen species (ROS) and demonstrated in bovine articular chondrocytes that ROS, such as the superoxide anion (O_2^-), peroxynitrite ($ONOO^-$) and the hydroxyl radical (·OH), play important roles in cartilage degradation [4–7]. In addition to these ROS, we demonstrated that hydrogen peroxide induced the apoptosis of chondrocytes [8, 9].

Nitric oxide (NO) also causes cartilage degradation by depressing the function (i. e., proteoglycan synthesis) and/or viability of articular chondrocytes [10, 11]. However, when we exposed chondrocytes to hypoxia, NO production was induced, which protected the chondrocytes from apoptosis resulting from oxidative stress [12]. Although these data demonstrated that NO induced by hypoxia plays a chondroprotective role, the precise underlying mechanism remains to be determined.

Hyaluronan (HA), which is a high molecular weight (10^4–10^7 Da) non-sulphated glycosaminoglycan (GAG) component of the extracellular matrix present in many tissues, consists of repeating disaccharide units of N-acetylglucosamine and d-glucuronic acid [13]. A family of enzymes responsible for the synthesis of HA, designated HAS for HA synthase, has been identified. Three distinct mammalian HAS gene products have been described, i. e. HAS 1-3 [14]. Of those, we found that only HAS2 mRNA was expressed in

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bovine articular chondrocytes [15]. Increased concentrations of HA have been found at sites of chronic inflammation [16], and the antioxidative effect of HA has recently been reported [17, 18]. The purpose of the present study was to determine if hypoxia induces HA synthesis by chondrocytes, and, if so, whether the hypoxia-induced HA synthesis is regulated by NO.

Materials and methods

Materials: Penicillin-streptomycin-amphotericin B, neomycin and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco (Grand Island, NY). Purified, high molecular weight (MW) HA (2.3, 1.8, 1.3, 0.8, 0.3 x 10^6 Da) was the kind gift of Chugai Co. Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT). Cetylpyridinium chloride was purchased from Sigma (St. Louis, MO). L-N-monomethylarginine (L-NMMA) and N-nitro-L-arginine methyl ester (L-NAME) were obtained from Dojindo (Kumamoto, Japan). Interleukin-1-beta (IL-1) and Texas Red conjugated horse anti-mouse (TI-2000) were purchased from Diaclone (Besancon, France) and Vector Laboratories (Burlingame, CA, USA), respectively. Polyclonal anti-iNOS antibody was purchased from Chemicon (Mississauga, ON, Canada).

Chondrocyte culture: Articular cartilage slices were taken from the condylar ridge of metacarpophalangeal joints of freshly slaughtered calves (approximately 10 months old). Chondrocytes obtained by enzymatic dissociation [19] were seeded at a density of 1 x 10^5 cells/cm^2. The culture medium was DMEM, supplemented with penicillin-streptomycin-amphotericin and 10 % FBS at 37 °C. The culture apparatus was supplied by SANYO Ltd (Osaka, Japan). Gas, composed of nitrogen with 5 % carbon dioxide and 1, 5, 10 or 21 % oxygen, was supplied by Takayasu Ltd (Osaka, Japan).

Generation of the ROS: In a total volume of 1 ml of 0.1 M potassium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride, ROS fluxes were generated by the reaction of 45 µM H_2O_2 and 5 µM $FeSO_4$. These reaction mixtures were incubated at 37 °C. Throughout the incubation, aliquots of the reaction mixture were extracted with phenol and chloroform (1/1) to stop the reaction [20]. Samples without a scavenger were used as a control.

Measurement of ROS: Reaction mixtures containing the chemiluminescent probe (L-012, 0.15 mg/ml) that reacts with ROS [21] were added and incubated for one minute. Chemiluminescence from the reaction mixture was detected by an ultra-sensitive photon-counting imaging camera (C-2400) equipped with a computer-assisted image processor (Argus 50, Hamamatsu Photonics, Hamamatsu, Japan). A few seconds after adding the L-012, the scintillating photonic images on a TV monitor rapidly increased. These dotted scintillating photonic images were digitally processed and accumulated in a video-frame memory through an image processor to determine ROS-generating sources [22].

Gel chromatography: Aliquots (100 µl) of the samples were chromatographed on Shodex OHpak SB806 HQ X2 (8 mm x 30 cm, Showa Denko, Tokyo, Japan). Fractions (0.5 ml) were eluted at 1 ml/min with 0.5 M sodium acetate (pH 5.6). Prior to use, the column was calibrated with blue dextran and phenol red [15].

NO production in cultured chondrocytes: Levels of NO in the medium were measured using the Greiss reaction and sodium nitrite as a standard [23]. Absorbance at 540 nm was determined using the Spectra plate reader (TECAN, Austria).

Determination of HA concentration: Levels of HA in the eluted samples were determined by a HA enzyme-linked immunosorbent assay (ELISA) Kit Echelon, Salt Lake City, UT) [24]. This system is a competitive ELISA assay in which the colorimetric signal is inversely proportional to the amount of HA present in the sample. Samples to be assayed are first mixed with the Detector, added to the HA ELISA Plate for competitive binding. An enzyme-linked antibody and colorimetric detection is used to detect the HA detector bound to the plate. The concentration of HA in the sample is determined using a standard curve of known amounts of HA. According to the manufacturer, the range of detection of this assay is 10~100 ng/ml.

Reverse transcription-polymerase chain reaction (RT-PCR): Total RNA was isolated using ISOGEN (Nippon Gene, Toyama, Japan) and subjected to RT-PCR using a One Step RNA PCR Kit (Qiagen, Germany). RT-PCR assays for HAS were performed in a T3 Thermocycler (Biometra, Germany). Primers were designed based on sequences for bovine HAS2: sense (5'-CTC GGA AGT AGG ACT TGC TCC AAC GG-3') and antisense (5'-ACA CAG CCT TCA GAG CAC TGG GAC-3'), both 472-bp products. RNA (1 *u*g) was reverse transcribed for 30 min at 50 °C and heat-denatured for 15 min at 95 °C. The cDNA was subjected to the following PCR cycles: 23 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for one minute. Each reaction contained 0.2 *u*M primer, 2.5 U Taq and 1 mM dNTPs. The PCR products were separated by electrophoresis through a 1.1 % agarose gel containing 0.5 *u*g/ml ethidium bromide and visualized by exposure to UV light. The GAPDH primers of sense (5'-CCA TCA CCA TCT TCC AGG AGC-3') and antisense (5'-GGA AGG CCA TGC CAG TGA GC-3') were used as controls for the total RNA applied.

Small interfering RNA (siRNA) and transfection: siRNA for iNOS was commercially synthesized and the FITC-tagged control siRNA was purchased from Qiagen-Xeragon (Germantown, MD). The sequence for iNOS siRNA was CCA GAC GAG CTT CTA CCT CAA. The control siRNA had no match to any eukaryotic sequence. Cells were seeded in a 12-well plate in the culture medium as described above at a density of 1 x 10^5 cells/cm^2 and incubated under normal growth conditions for four days. Transfection of siRNA was carried out using the RNAi Starter kit (Qiagen, Germany). Briefly, cells were washed with medium and then kept in 600 µl of the medium until transfected. Meanwhile, siRNA (2 µg) was diluted in the appropriate volume of the culture medium to give a final volume of 100 µl each in separate sterile tubes and mixed by vortexing. For complex formation, RNAiFect transfection reagent (12 µl) was added to the diluted siRNA and incubated at room temperature for 10 min. The formed complex (100 µl) was added to the cells, prepared as described above, and incubated under normal growth conditions for 48 h before hypoxic treatment. Cells were cultured for an additional 24 h under hypoxic conditions (5 % O_2).

Immunofluorescence: After siRNA transfection, cells were incubated under normal growth conditions for 48 h. Cells were fixed for 10 min in 3.7 % paraformaldehyde. Following three 5-minute washes with PBS, they were blocked for 15 min in antibody diluents. Cultures were incubated for 1 h with primary antibody, i. e. polyclonal anti-iNOS antibody. Following washing with PBS, the monolayer was incubated in the dark for 30 min with Texas Red conjugated second antibody. Texas Red was excited at a wavelength of 543 nm and collected through a long pass filter (560LP). Texas Red was assigned to the red channel respectively of the generated RGB image [25]. To determine the effectiveness of transfection, non-transfected cells were incubated with IL-1 (10 ng/ml) for 6 h as a positive control.

Western blotting analysis for iNOS protein: Cells transfected with iNOS-siRNA were incubated under normal growth conditions for 48 h. Cells were lysed with CelLytic-M Mammalian Cell Lysis/Extraction Reagent (Sigma, St Louis, MO). The protein concentration was determined by the bicinchoninic acid (BCA) method. The samples from each experiment (20 µg per lane) were separated by 7.5 % SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. After incubation with 5 % BSA in 20 mM Tris-buffered saline/0.05 % Tween 20, the membranes were incubated with primary mouse monoclonal antibody to iNOS, diluted 1:2500 (BD Biosciences, Franklin Lakes, NJ) and monoclonal antibody to β-actin, diluted 1:5000 (Sigma, St. Louis, MO), overnight at 4 °C. Membranes were washed and then incubated for 1 h with secondary mouse monoclonal antibody diluted 1:10000 (Amersham, Piscataway, NJ). iNOS protein on the membrane was detected with the ECL system (Amersham, Piscataway, NJ).

Statistical analyses: Results are presented as the mean ± standard deviation (SD). The Student's t test was used for statistical assessments. A level of $p < 0.05$ was considered significant.

Results

We first examined the antioxidant properties of HA. The hydroxyl radical, which is one of the ROS, was generated via the reaction between H_2O_2 and Fe^{2+}: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + {}^{-}OH$. The kinetics of the generation of the hydroxyl radical was determined using the chemiluminescence probe, L-012. The photointensity of the chemiluminescence from the reaction mixture increased rapidly after mixing H_2O_2 and Fe^{2+}, but returned to baseline levels within three minutes. The addition of HA (MW: 0.8 x 10^6 Da) inhibited the ROS-enhanced photointensity (Fig. 1). We further examined the antioxidant properties of HA and found that HA inhibited the ROS-enhanced photointensity in a MW-dependent fashion (Fig. 2).

To determine the effect of oxygen tension on chondrocyte metabolism, bovine articular chondrocytes were cultured for 12 h at O_2 concentrations of 1, 5, 10 or 21 %. Those chondrocytes maintained at 5 % O_2 had the highest levels of HA in the medium. Medium HA levels were significantly lower in the 21 % O_2 cultures when compared to the 5 and 10 % O_2 cultures ($p < 0.01$) (Fig. 3). We also measured examined alterations in the molecular weight of HA secreted in the medium from chondrocytes cultured with different oxygen tensions (21 % and 5 %) for 12 h. The medium was collected and subjected to gel chromatography as described above. There were no differences in the molecular weight of HA synthesized by the chondrocytes cultured with different oxygen tensions (data not shown).

We next examined whether NO was involved in the hypoxia-induced increase in HA synthesis, as demonstrated by significantly enhanced levels of HA in the medium that were found with exposure to 5 % O_2 (Fig. 3). We measured NO levels in the medium and found that hypoxia clearly enhanced NO levels in the medium [21 % O_2: 13.2 ± 0.9, 10 % O_2: 18.2 ± 0.8*, 5 % O_2: 21.0 ± 1.0*, 1 % O_2: 14.2 ± 1.1 µmol/l. Data are expressed as the mean ± SD (n = 8, * $p < 0.05$ vs. 21 % O_2)]. When the NO inhibitors, L-NMMA (1 mM) and L-NAME (1 mM), were added to this system, the hypoxia-enhanced HA levels in the medium were significantly inhibited (Fig. 4). In these samples, the quantity of RT-PCR products generated from HAS mRNA was enhanced in bovine chondrocytes exposed to hypoxia for 12 h. The NO inhibitors, L-NMMA (1 mM) and L-NAME (1 mM), also inhibited the expression of HAS mRNA that was enhanced under hypoxic culture conditions (5 % O_2) (Fig. 5).

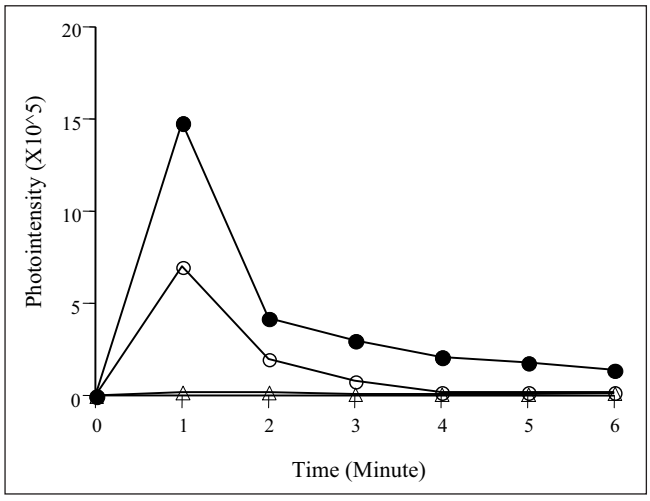

Fig. 1. Kinetics of the generation of the hydroxyl radical. Hydroxyl radicals were generated by the reaction of 45 µM H_2O_2 and 5 µM $FeSO_4$. Using L-012 (0.15 mg/ml), the photointensity of the chemiluminescence from the reaction mixture was measured. Triangle:H_2O_2, Closed cicle: $H_2O_2 + Fe^{2+}$, Open circle: $H_2O_2 + Fe^{2+}$ + hyaluronan (HA). (100 µM, molecular weight (MW): 0.8 x 10^6 Da). One representative experiment of three is shown.

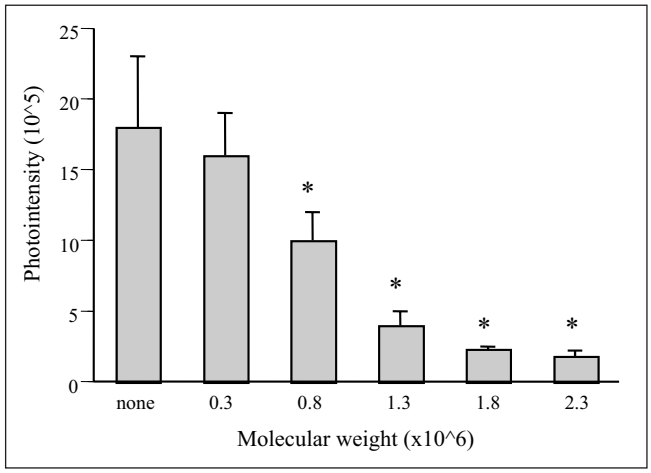

Fig. 2. The effect of hyaluronan on the generation of hydroxyl radicals. Hydroxyl radicals were generated via the reaction between H_2O_2 and Fe^{2+} in the absence or presence of HA (100 µM) of varying molecular weights (0.3 to 2.3 x 10^6 Da). Using L-012, the photointensity of the chemiluminescence at 60 s after the addition of $FeSO_4$ was measured. Data are expressed as the mean ± standard deviation for four samples. Representative results of two different experiments are shown. *:$p <$ 0.01 vs. control (none).

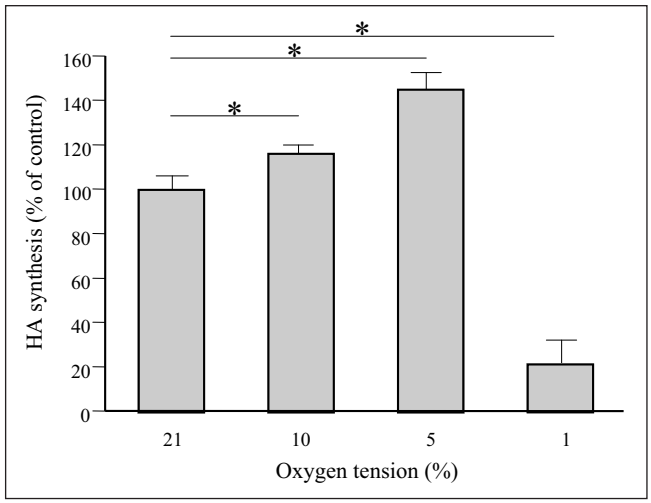

Fig. 3. Hyaluronan synthesis. Bovine chondrocytes were cultured at different oxygen tensions (21 %, 10 %, 5 % or 1 %) for 12 h. The levels of HA in the medium were measured. The data are expressed as the percent of the control chondrocytes, i.e. those cultured at 21 % O_2. Columns and bars represent the mean and standard deviation, respectively. *$p <$ 0.01, n = 10.

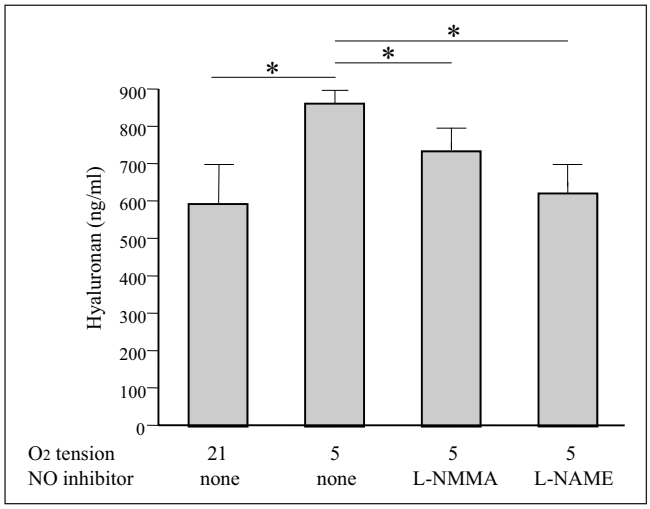

Fig. 4. The role of NO in hypoxia enhanced hyaluronan synthesis. Bovine chondrocytes were cultured with 21 % O_2 or 5 % O_2 in the absence or presence of the NO inhibitors, L-NMMA (1 mM) or L-NAME (1 mM), for 12 h. Columns and bars represent the mean and standard deviation, respectively. *p < 0.01, n = 10.

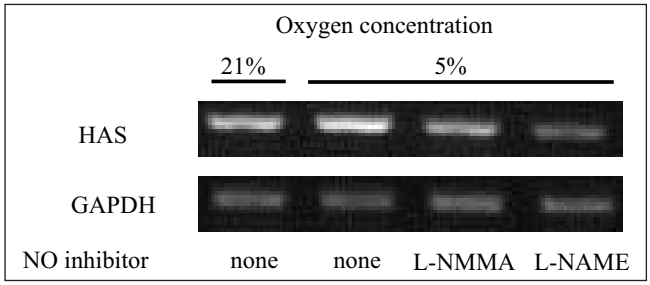

Fig. 5. Expression of hyaluronan synthase (HAS) mRNA. Bovine chondrocytes were cultured under normoxia (21 % O_2) and hypoxia (5 % O_2) for 12 h in the presence or absence of the NO inhibitors, L-NMMA (1 mM) or L-NAME (1 mM). Total RNA was isolated and subjected to RT-PCR for HAS2 and GAPDH.

In order to determine if the increased levels of HA seen after hypoxia were a result of the increased levels of NO, double-stranded short interfering RNA oligonucleotides of NOS (iNOS-siRNA) were directly transfected into the chondrocytes 24 h prior to exposure to hypoxia (5 % O_2). Greater than 80 % of the cells were transfected (data not shown). To confirm that transfected iNOS-siRNA effectively inhibited iNOS synthesis, we performed the histochemistry using confocal microscopy. Detection of iNOS antigens decreased with iNOS-siRNA, whereas exposure to IL-1 clearly enhanced iNOS expression (Fig. 6). In addition, we assessed iNOS protein levels using Western blot analysis. The level of iNOS protein expression decreased with transfected iNOS-siRNA, whereas exposure to IL-1 enhanced iNOS protein expression (Fig. 7). After 12 h of hypoxia, the hypoxia-enhanced NO levels were significantly decreased by iNOS-siRNA transfection. Levels of HA in the medium were also measured. The results show that iNOS knockdown resulted in a complete inhibition of the hypoxia-induced upregulation of HA levels in the medium (Table 1).

Table 1. Effect of siRNA targeting iNOS treatment on NO and HA expression

Oxygen tension (%)	iNOS-siRNA	NO synthesis (μmol/l)	Hyaluronan (ng/ml)
21	(–)	13.2 ± 0.9	972 ± 42
5	(–)	21.0 ± 1.0*	1324 ± 60*
5	(+)	14.8 ± 1.1[a]	610 ± 88[a]

NO and HA expression in the presence of iNOS-siRNA. Chondrocytes were transfected with iNOS-siRNA and subjected to hypoxic treatment. Values are the mean and SD, respectively (n=10). *p<0.01 vs. 21 % (–). [a]p<0.01 vs. 5 % (–).

Discussion

One of the ways that ROS degrade cartilage is by damaging matrix components [26]. Several in vitro studies have reported the degradation of cartilaginous tissue slices by the direct attack on proteoglycan and collagen molecules by ROS. Specifically, the incubation of acid-soluble type I collagen with superoxide anion radicals degrades the collagen and prevents the formation of fibrils by this collagen [27]. In the presence of oxygen, the hydroxyl radical degrades collagen and modifies its amino acid composition. Hydroxyl radical also attacks on the polysaccharide backbone of HA [7].

In the present study, the hydroxyl radical was generated by the reaction of H_2O_2 and $FeSO_4$. We showed that HA reduced the formation of ROS in a molecular weight-dependent fashion. Since HA is depolymerised by the direct oxidative cleavage at the glycosidic bonds, the mechanism of which have now been fully elucidated [28], it is clear that high molecular weigh HA with many glycosidic bonds is more effective to neutralize the ROS than lower one [29]. Our data indicating the antioxidant effect of HA is consistent with other systems [17, 18]. Inflammation of the articular joints is accompanied by a decrease in the viscosity of the synovial fluid in which HA is the major macromolecule and imparts viscosity. It has been hypothesized that the production of the hydroxyl radical may be responsible for the degradation of the HA present in rheumatoid synovial fluid [30, 31]. This may in part explain why the administration of exogenous high molecular weight HA in patients with arthritis slows disease progression [32].

Another way that ROS contribute to the degradation of articular cartilage is by the induction of chondrocyte apoptosis. Chondrocyte death is now considered to be an important factor contributing to the breakdown of the extracellular matrix in joint diseases. Decreased chondrocyte viability impairs self-repair in cartilage and may in fact accelerate the progression of the lesion. Although NO has long been considered to be the primary inducer of chondrocyte apoptosis [10, 11], it has become clear that NO by itself cannot initiate apoptosis [26]. In fact, NO could be anti-apoptotic, primarily when the intracellular antioxidant level is very low [33]. We previously demonstrated the induction of NO by

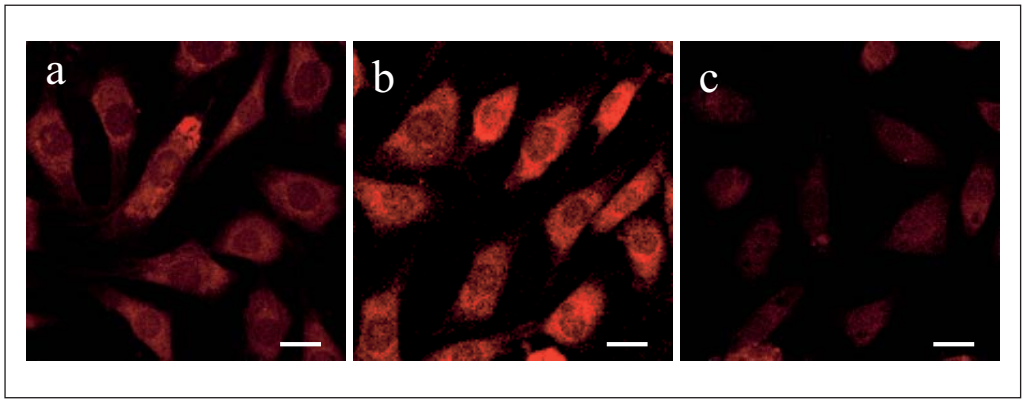

Fig. 6. Immunofluorescence imaging for the detection of iNOS antigens. After siRNA transfection, cells were incubated with polyclonal anti-iNOS antibody. Following washing with PBS, they were incubated with Texas Red conjugated second antibody. (a) Untreated chondrocytes. (b) Following exposure to IL-1 (10 ng/ml) for 6 h, iNOS expression in non-transfected cells was markedly increased. (c) Specific inhibition of iNOS expression in the chondrocytes by iNOS-siRNA. Bar = 20 µm.

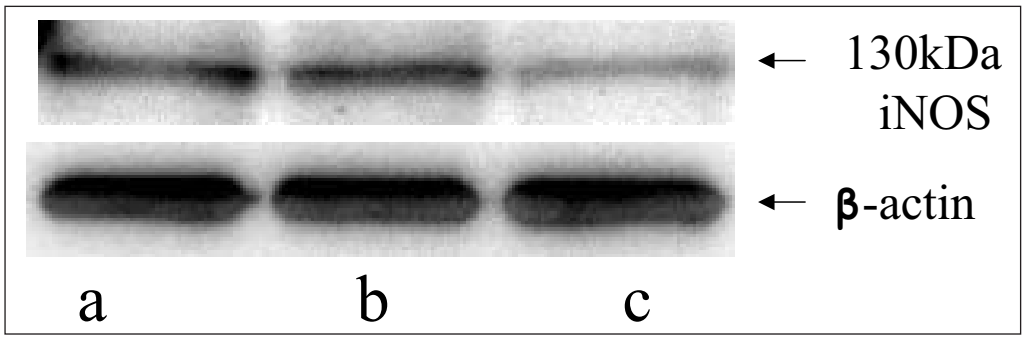

Fig. 7. Representative Western blot demonstrating expression of iNOS protein. Cells transfected with iNOS-siRNA were incubated under normal growth conditions for 48 h. (a) Untreated chondrocytes. (b) Following exposure to IL-1 (10 ng/ml) for 6 h, expression of iNOS protein in non-transfected cells was markedly increased. (c) Specific inhibition of iNOS protein in the chondrocytes by iNOS-siRNA.

hypoxia, which attenuated the ROS-induced chondrocyte damage [12].

In the present study, chondrocytes maintained at 5 % O_2 achieved the highest level of HA production. On the other hand, there was a drastic decrease in HA synthesis at 1 % oxygen. We examined the effect of hypoxia on cell damage using annexin V-FITC and propidium iodide and found there was no cell damage at this condition (data not shown). These data indicate that chondrocytes are metabolically less active when cultured at 1 % oxygen and a similar effect has been seen in cultures of bovine articular cartilage explants [34]. HA levels in the medium were significantly lower in 21 % O_2 cultures when compared to the 5 % and 10 % O_2 cultures. Hypoxia also enhanced HAS expression. Hypoxia inducible factor 1 (HIF-1) is a transcription factor that regulates cellular responses to hypoxia. It is possible that induced HIF-1 with hypoxia enhanced NO synthesis because one of the currently known HIF-1 target genes is iNOS [35, 36]. However, one important question was remained, how NO impacts on HA synthesis. Although further study is necessary, it is clear that this signaling pathway play an important role in the pathophysiology of joint disease. Recently, expression of HIF-1 was reported in the human articular cartilage of osteoarthritis [37].

Because HA has diverse effects depending on its molecular weight [38], we investigated the effects of hypoxia on alterations of the molecular weight of HA. There were no differences in the molecular weight of HA synthesized by chondrocytes exposed to hypoxia. In a previous study, we showed enhanced proteoglycan synthesis by chondrocytes cultured with 5 % O_2 [12]. To summarize, it is clear that reduced oxygen tension increased the biosynthetic activity of chondrocytes. Interestingly, NO inhibitors (L-NMMA and L-NAME) inhibited hypoxia-enhanced HA levels in the medium; this phenomenon was regulated at the mRNA level.

RNA-interference is a technology that can be used to study the effects of gene inhibition in the context of the cell. One method of RNA-interference is to use small interfering RNAs (siRNA) with a sequence complementary to the gene of interest. When short sequences, approximately 21 nucleotides in length, are present in the cell, the mRNA with a complementary sequence is degraded and, therefore, translation does not occur [39, 40]. Gene expression can be knocked down by 90 % or more and can thereby achieve results similar to a genetic knockout. In the present study, we used histochemistry to confirm that the depletion of iNOS is indeed a result of siRNA transfection. We showed that when expression of NO synthase was knocked down with siRNA, levels of HA in the medium decreased as well.

These data indicate that that NO is responsible for the defense of chondrocytes by inducing the synthesis of the antioxidant HA which helps the removal of ROS. Although OH radicals were produced and studied in the present study, we could not exclude a more direct role of NO in the removal of some ROS, e. g. reaction with superoxide. In this context, peroxynitrite, which is produced with the reaction between superoxide anion and NO, can cause the damage of chondrocytes [5] and would also be removed by the high molar mass HA.

Taken together, the endogenous NO synthesis plays an important role in hypoxia-enhanced HA synthesis.

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