Changes in the production and the effect of nitric oxide with aging in articular cartilage

An experimental study in rabbits

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ABSTRACT – We studied the production and the effect of nitric oxide (NO) in articular cartilage from rabbits of various ages. 40 New Zealand white rabbits in 4 age groups (1 month, 6 months, 1 year, and 3 years of age with 10 rabbits in each group) were used. Basal and induced levels of NO were lower in cultured chondrocytes from older rabbits. Exogenous NO administration suppressed the proliferative activity of chondrocytes to a greater degree in younger rabbits. Immunohistochemistry showed that older rabbits had fewer eNOS positive chondrocytes. Our findings imply that the age-related change in NO in articular cartilage does not have a relevance to increased NO production in osteoarthrosis.

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Nitric oxide (NO) is a free radical gas that mediates a variety of biological pathways and controls many physiological phenomena inside the cells of mammals. NO is generated by the conversion of L-arginine to L-citrulline in an oxidation reaction catalyzed by nitric oxide synthase (NOS) (Evans et al. 1995). External stimuli, such as lipopolysacharide (LPS) or cytokines like tumor necrosis factor (TNF)-α, activate nuclear factor kappa B (NFκB), a transcription factor that promotes the synthesis of inducible NOS (iNOS). NO then activates soluble guanylyl cyclase by binding to the prosthetic heme moiety of the enzyme. The resulting cyclic guanine monophosphate (cGMP) in turn functions as the effector molecule (Taylor et al. 1998). NO also activates cyclooxygenase, producing a burst of prostaglandin E2 synthesis (Blanco and Lotz 1995, Manfield et al. 1996).

In articular cartilage, biosynthesis of nitric oxide is also triggered by cytokines and LPS (Stadler et al. 1991). NO thus produced suppresses the proliferation of chondrocytes and the production of proteoglycan (Blanco et al. 1995a). An overdose of NO induces apoptosis of chondrocytes in the absence of other oxygen radicals (Blanco et al. 1995b, Hashimoto et al. 1998). The amount of NO synthesis varies with the zone of articular cartilage, superficial layers produce more NO (Fukuda et al. 1995, Hayashi et al. 1997, Hauselmann et al. 1998).

Recently, NO has been the subject of many studies devoted to aging (Mollace et al. 1995, Tschudi et al. 1996, McCann 1997, Chou et al. 1998). Articular cartilage, in particular, changes greatly during human life. However, hardly any information is available concerning changes in the production and action of NO in articular cartilage with aging. Therefore, we aimed to study the age-related changes in articular cartilage as regards the production of nitric oxide and its effect on cell proliferation.

Using an animal model, we measured the basal and induced production of NO from cultured chondrocytes with a nitrite assay and detected endothelial NOS (eNOS) from articular cartilage with immunohistochemical staining. We also measured the change in the proliferative activity of chondrocytes after giving exogenous NO with a nonradioisotopic proliferation assay.

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Animals and study design

We studied 40 New Zealand white rabbits. 20 of them were used for chondrocyte isolation and cell culture, and the remaining 20 for immunohistochemical analysis. The animals in both groups were subdivided into 4 age groups (1 month, 6 months, 1 year, and 3 years of age), each comprising 5 rabbits.

The rabbits were anesthetized and prepared for sterile surgery. A straight incision was made over both knee joints, the patella was everted, and articular cartilage removed from the femoral condyles. To obtain the specimens for immunohistochemistry, right distal femora were removed en bloc, and immersed in 4% paraformaldehyde solution. The rabbits were then killed with an overdose of barbiturate given intravenously. The experiments were approved by the University Committee for Animal Experimentation.

Cell culture

Harvested articular cartilage was sliced to a size of 2 mm, washed three times with PBS, and incubated in 0.5% trypsin phosphate buffered saline (PBS) solution at 37 °C for 30 minutes. It was again washed three times with PBS, and incubated in Ham's F-12 solution (Gibco, Green Island, NY, USA), containing 0.05% clostridial collagenase (Sigma, St. Louis, MO, USA) at 37 °C for 16 hours. The supernatant was removed and centrifuged at 200 times the force of gravity for 5 minutes. The pellets obtained from this were washed three times with PBS. The cell population was determined with a hemocytometer. The cells were cultured in 96-well plates in Ham's F-12 medium (200 μL per well) containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-gentamicin-amphotericin) at 37 °C in a humidified 5% CO_2 atmosphere. Individual wells were populated by 2×10^5 cells for nitrite assay, and 2×10^4 cells for proliferation assay.

Nitrite assay

Nitrite, a stable end-product of NO, was measured from the media at 72 hours of culture. Two subsets were prepared for chondrocytes in each assay. In the first subset, we measured basal secretion with no additive. In the second subset, 0.2 μg of LPS, inducer of NO, was administered to give a final concentration of 1 μg/mL 24 hours before measurement. Measurement entailed spectrophotometric analysis based on the Griess reaction. Briefly, 100 μL of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamide in 5% phosphoric acid) was added to 100 μL of cell culture medium. After incubating for 30 minutes, the optical density was measured at 540 nanometers with a spectrophotometer. Nitrite concentration was measured by comparison with the optical density of standard solutions of sodium nitrite prepared in Ham's F-12 medium, and the final basal nitrite level was obtained by subtracting the nitrite concentration of the control well (which had been filled with medium only) from that of the first subset. The induced NO level was defined as the difference between the value from the first subset (no additive) and the second subset (LPS added). The cell culture and assays were done in triplicate, and the average of the triplicates was used as the representative value.

Proliferation assay

To observe cell growth, we used a cell proliferation ELISA kit (Boehringer Mannheim, Mannheim, Germany). The assay exploited the incorporation of 5-deoxybromouridine (BrdU), a pyrimidine analogue, into DNAs of the proliferating cell. Anti-BrdU antibody conjugated with peroxidase was used for immunodetection. Two subsets were prepared for chondrocytes from each rabbit. The first subset had no additives. In the second subset, sodium nitroprusside (SNP) (Sigma, St. Louis, MO, USA) was given to achieve a final concentration of 1 mM 24 hours before measurement. At 72 hours of culture, BrdU was added to the wells to obtain a final concentration of 10 μM, and incubated for 6 more hours. The cells were then fixed with 70% ethanol. 100 μL of anti-BrdU antibody conjugated with peroxidase was applied for 2 hours at room temperature, and then washed three times with PBS. 100 μL of tetramethyl benzidine (TMB) solution was added as a substrate. After 30 minutes, the optical density was measured at 370 nanometers (reference filter 490 nanometers). From these readings, the optical density of the negative control, which followed identical procedures except for BrdU administration, was subtracted from the

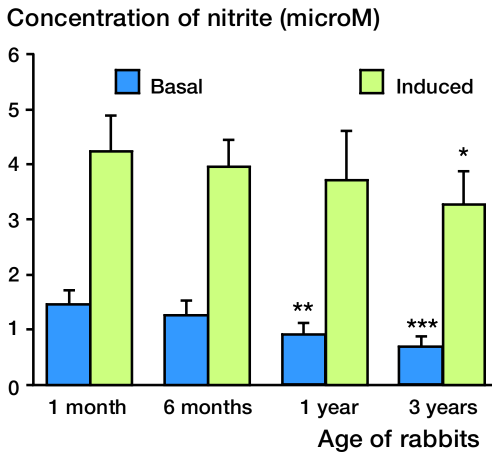

Figure 1. Levels of nitrite in cultured lapine chondrocytes. Basal levels and LPS-induced levels are shown. The levels of nitrite were measured after 72 hours of culture. LPS, inducer of NO, was given to a final concentration of 1 μg/mL 24 hours before measurement. The values are expressed as means with standard deviations (n 5, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. corresponding levels of 1-month-old rabbits, Basal: basal nitrite level, Induced: induced nitrite level).

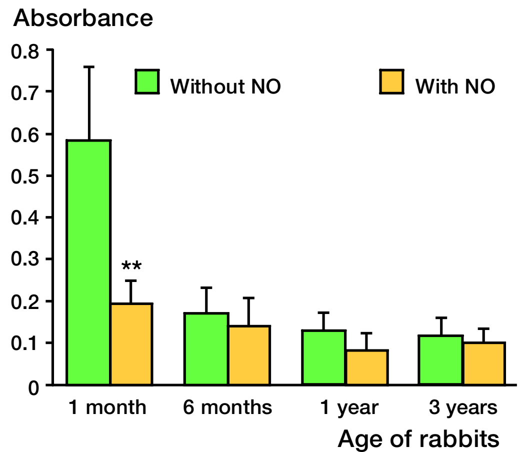

Figure 2. Proliferative activity of the chondrocytes after exogenous NO. Optical density was measured at 72 hours of culture. SNP was givento achieve a final concentration of 1 mM 24 hours before measurement. The values are expressed as means with standard deviations (n 5, ** $p < 0.01$ vs absorbances of the same age group without SNP, without NO: no additive, with NO: SNP given).

value of each subset. The cell culture and assays were done in triplicate. The average of the triplicates was used as the representative value.

Immunohistochemistry

The specimens were immersed in 4% paraformaldehyde for 12 hours, then decalcified in a solution of 0.1% w/v ethylenediaminetetraacetate (EDTA) at room temperature for 7 days, embedded in paraffin. The sections were cut in the sagittal plane, deparaffinized with xylene, rehydrated with decreasing concentrations of ethanol solution and rinsed three times for 5 minutes each with 0.01M PBS (pH 7.2). Sections were processed with 0.25% trypsin for 15 minutes, and 3% hydrogen peroxide to suppress intrinsic peroxidase activity. Blocking solution containing normal rabbit serum was added to the section for 30 minutes. The sections were incubated with monoclonal anti-eNOS mouse antibody (Transduction Laboratory, Lexington, KY) at an optimal dilution in PBS with 1% w/v bovine serum albumin for 2 hours. They were then rinsed with PBS three times for 5 minutes each, and incubated with biotinylated anti-mouse immunoglobulin (Dako, Copenhagen, Denmark) for 1 hour. Streptavidine-peroxidase complex was then applied for 30 minutes, and the specimens rinsed with PBS. Substrate solution (3% 3-amino-9-ethylcarbazol as chromogen in 0.01% Tris buffer (pH 7.6) containing 0.005% hydrogen peroxide) was applied. Hematoxylin was used for counterstaining. Sections, in which the primary antibody had been omitted, were used in each run to assess nonspecific binding of the secondary antibody.

Statistics

Descriptive statistics were used to determine group means and standard deviations. Statistical analysis was done with analysis of variance (ANOVA) and the Student's t-test (two-tailed). Significance was set at $p < 0.05$.

Results

Nitrite levels

The basal nitrite concentration declined with the rabbits' age. Chondrocytes from 1-month-old rabbits had twice the level (1.4 μM, SD 0.26) as those of 3-year-old rabbits (0.69 μM, SD 0.18). Chondrocytes from 6-month-old and 1-year-old rabbits showed intermediate values—i.e., 1.3 μM (SD 0.29) and 0.90 μM (SD 0.22), respectively. The differences between 1-month-old and 1-year-

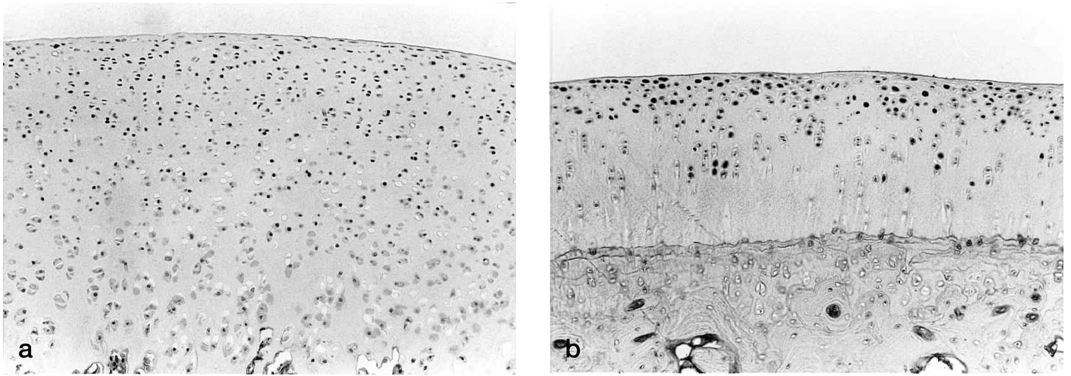

Figure 3. Immunohistochemical staining of the articular cartilage ×100. a. eNOS in 1-month-old rabbit b. eNOS in 3-year-old rabbit.

old rabbits (p = 0.007) and between 1 month-old and 3-year-old rabbits were statistically significant (p = 0.0009). LPS increased the nitrite level in all groups ($p < 0.0001$). The induced nitrite levels also tended to fall gradually with age of the animals. The nitrite level in 3-year-old rabbits was lower than that in 1-month-old ones (p = 0.02) (Figure 1).

Proliferation assay

The absorbance (proliferative activity of the chondrocytes) was highest in 1-month-old rabbits, falling steeply in older ones. Exogenous NO dramatically reduced the proliferative activity in chondrocytes from 1-month-old rabbits (p = 0.005). The reduction was much less in chondrocytes from other age groups (Figure 2).

Immunohistochemistry

Positively-stained cells for eNOS were found in all age groups, and the percentage of positive cells fell with age. eNOS protein tended to be located in the superficial layer of articular cartilage (Figure 3).

Discussion

In this study, we tried to determine the change in production and action of nitric oxide with aging. A previous study found that basal production of nitric oxide was more than twice as high in articular chondrocytes from 1-month-old rats as in cells from older rats (Khatib et al. 1998). Another study of human articular chondrocytes showed an age-dependent decline in NO production in response to interleukin-1 (Hauselmann et al. 1998). The results of our study with lapine chondrocytes were similar, although the nitrite level showed considerable individual variations in rabbits.

Basal nitrite measurements reflect considerable constitutive NOS activity in the articular cartilage, at least in rabbits. Since NO is mainly associated with catabolism in articular cartilage and since it induces apoptosis of chondrocytes, the finding that NO production is more abundant in younger cartilage raises questions as to the exact role of NO. The action of NO is known to vary with the amount produced. A small amount of NO produced by constitutive NO may play a physiological role, but bursts of NO production by inducible NOS in response to noxious stimuli increase inflammation (Amin and Abramson 1998). Stimulation of chondrocytes by LPS increased the amount of nitrite produced. The induced nitrite levels fell less with aging, suggesting that enough iNOS can still be produced by stimulation in older animals.

Our immunohistochemical findings showed a predilection of eNOS for the superficial zone. This accords with recent studies indicating that chondrocytes from the superficial zone of articular cartilage synthesize NO 2–3 times more than those from the deep zone (Hayashi et al. 1997, Hauselmann et al. 1998). Therefore, NO seems to be produced mainly by superficial chondrocytes in articular cartilage.

When NO was given exogeneously, the suppression of cell proliferation was more marked in chondrocytes from younger rabbits. The reduction in cell proliferation was much less in older ones. The relative insensitivity of senescent cells to NO donors may be due to their reduced capacity to pro-

duce cGMP, as shown by Khatib et al. (1997), or also to their already low proliferative activity.

Our findings differ from those in osteoarthrotic cartilage, where increases in production are ascribed to the pathogenesis of osteoarthrosis (Amin and Abramson 1998, Melchiorri et al. 1998, Salvatierra et al. 1999). However, the results of our study and others show that NO production declines in normal senescent articular cartilage (Khatib et al. 1998). In our proliferation study, we also found that chondrocytes from older rabbits are much less sensitive to suppression by NO. Since our study is based on a rabbit model and human cartilage has a much wider age span and different structure, no definite conclusions regarding human joint disease can be drawn. However, our data suggest that an increase in NO production in osteoarthrosis may not be related to the change with age in NO in articular cartilage, but instead may be a secondary change occurring after some other cause.

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