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Effects of ageing and arthritic disease on nitric oxide production by human articular chondrocytes

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Abbreviations: OA, osteoarthritis; NO, nitric oxide; IL-1β, interleukin-1β; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; AG, aminoguanidine; iNOS, inducible NO synthase; ncNOS, neuronal NOS; eNOS, endothelial NOS

Abstract

Nitric oxide (NO) has been considered as an important mediator in inflammatory phases and in loss of cartilage. In inflammatory arthritis, NO levels are correlated with disease activity and articular cartilage is able to produce large amounts of NO with the appropriate inducing factor such as cytokines. The old animals are shown to have a greater sensitivity to NO than young animals. This study evaluated the basal production of NO in normal and OA-affected chondroyctes from young and old patients and compared the levels of NO formation in response to IL-1β. The results showed that the basal levels were 7-fold higher in old chondrocytes than those of young cells. However, the IL-1β induced NO production was seen to decrease with age. Aminoguianidine (AG), a competitive inhibitor of iNOS, inhibited NO formation completely in both chondrocytes from young and old individuals. However, at the same concentration of AG it caused partial inhibition of NO and iNOS formation in chondrocytes from OA-affected individuals. In addition, although the IL-1β induced NO production was much lesser than that of young chondorycytes, the inhibition of collagen production by IL-1β was prominent in old chondroyctes and OA-affected chondroyctes. These results suggest that age-related differences in the regulation of NO production and collagen production, which may affect the ageing cells and osteoarthritic changes in some way.

Keywords: ageing, human articular chondrocytes, interleukin-1, nitric oxide, osteoarthritis

Introduction

Articular cartilage in adult animals and humans undergoes a number of age-related changes, including a decrease in the number of chondrocytes (Barnett *et al.*, 1963; Mitrovic *et al.*, 1983) and an increase in the degradation of matrix components (Mitronic and Riera, 1992). Such age-related degeneration of articular cartilage could be a major risk factor for the development of osteoarthritis (OA) (Stefanovic-Racic *et al.*, 1993; Jang and Murrel, 1998), but the mechanism by which ageing is involved in the etiology of OA remains unclear.

Nitric oxide (NO) is known to play an important role in the damage of cartilage in arthritic conditions. As first shown by Stadler *et al.* (1991), articular chondrocytes produce large amounts of the free radical NO in response to cytokines such as interleukin-1β (IL-1β). NO inhibits chondrocyte proliferation by IL-1 (Blanco *et al.*, 1995), and endogenously generated NO inhibits the synthesis of proteoglycan (Stefanovic-Racic *et al.*, 1997) and collagen (Murrel *et al.*, 1995; Cao *et al.*, 1997) by chondrocytes. Therefore NO production in articular chondrocytes may contribute to chondrocyte ageing, and may be an important factor in the pathophysiology of OA. However, to date, there is no information on the differences in NO production by chondrocytes from young and old articular cartilage, and osteoarthritis-affected cartilage.

In the present study, we compared the ability of human articular chondrocytes obtained from young and old patients without OA, and from OA-affected cartilage, to synthesize NO in response to IL-1β. We also have investigated that NO mediates the different effects of IL-1β upon collagen type II production by young, old and OA-affected chondrocytes.

Materials and Methods

Cartilage sampling and chondrocyte culture

Human articular cartilage from femoral condyles was

obtained during total knee replacement from knee fracture patients of over 60 years of age (old cartilage) and from the individuals under 40 years of age (young cartilage) with no history of joint disease respectively. OA-affected cartilage was obtained from OA patients, between 60 and 65 years of age, undergoing knee replacement surgery.

Macroscopically normal, nonarthritic-cartilage from chondyles was dissected under sterile conditions and chondrocytes were isolated from the harvested slices of articular cartilage by digestion with trypsin, hyaluronidase and collagenase as previously described (Min *et al.*, 1998). Chondrocytes were seeded into 100 mm plates at a density of 4×10^5 cells in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 units of penicillin/ml and 100 μg streptomycin/ml. Following adherence, the cells were allowed to reach confluence. At that time, the monolayers were trypsinized, and the cells plated out at a density of 5×10^4 cells/well in 96-well tissue culture plates in 200 μl culture medium and allowed to adhere for 24 h.

Measurement of NO production

NO formation was detected as NO_2^- accumulation in the culture supernatants, as determined by the Griess reaction using sodium nitrite as standard (Green *et al.,* 1982). Briefly, 50 μl of culture supernatant was incubated with 50 μl 1% sulfanilamide, 0.1% *N*-1-naphthylethylenediamide dihydrochloride in 25% H_3PO_4 at room temperature for 5 min. OD was measured at 570 nm. As evaporation of the medium can falsely elevate nitrite concentrations, blank plates containing the media under similar conditions, with no added cells, were used as controls to determine any evaporation effects. Aminoguanidine (AG) was used as the control to validate the NO assay system. Each experiment used duplicate samples and each was repeated four times. Pooled data were used to generate the data presented here (n=4). Student's t-test for unpaired data was used for statistical analysis and $P \leq 0.05$ was considered significant.

Measurement of chondrocytic phenotype

To observe phenotypic differences between the young, old and OA-affected chondrocytes, we analyzed the expression of collagen type II by Western blotting using a mouse anti-human collagen type II specific antibody (Chemicon). Briefly, the protein was extracted with lysis buffer (40 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% NP-40, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride) and electrophoresis was conducted with 8% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to a 0.22-nm nitrocellulose membrane with a transblot apparatus (Bio-Rad). The blot was first incubated with the collagen type II Ab (diluted 1:2000 in 3% nonfat dry milk) and followed by a peroxidases-labeled sheep anti mouse IgG (Caltag). Visualization was achieved by a horseradish peroxidase immunostaning system (Armersham-Pharmacia).

Results and Discussion

It was found that there were significant differences in NO production between young and old articular chondrocytes, and OA-affected chondrocytes. First passage of cultured articular chondrocytes prepared from normal young and old cartilage and OA-affected cartilage produced NO differentially. NO is detected as the stable oxidation product, nitrite (NO_2^-), in the medium, reflecting the presence of an activity of inducible NO synthase (iNOS or NOSII) in these cells. It was found that basal NO production in articular chondrocytes was increased with the age of the cells (Figure 1). The basal level of constitutive NO production in old chondrocytes of weight bearing and non-weight bearing regions was increased by >5- and >15-fold, respectively, compared with that of young cartilage. In addition to the higher levels of NO in old cartilage, the NO level in the chondrocytes from OA affected-cartilage was also 7-fold more than the basal level in the young chondrocytes. These findings of elevated NO levels in the human articular chondrocytes from the aged and OA-affected cartilage are in contrast to reports of an age-dependent decline in the basal NO production on the rat articular chondrocytes (Khatib *et al.,* 1998). These disparate results indicate that species-specific differences in NO responses should be carefully considered.

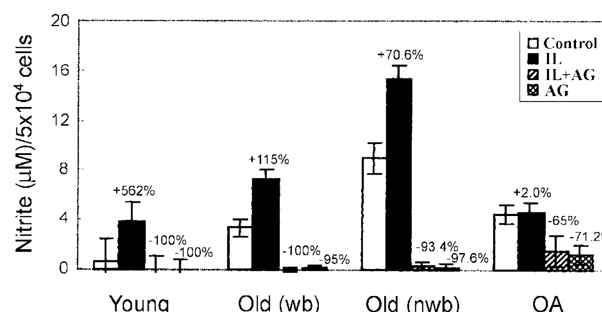

Figure 1. The effect of IL-1β on NO production by human articular chondrocytes. Chondrocytes were isolated from young articular cartilage, weight-bearing (wb) sites of old cartilage, non-weight bearing (nwb) sites of old cartilage, and OA-affected cartilage. Chondrocytes cultured for 48 h with culture medium alone (Control), or with 5 ng/ml IL-1β, aminoguanidine (AG; 1 mM). Nitrite in the culture supernatants was measured with the Griess reagent. Bars show the mean±S.D. of 4 separate experiments, each performed in duplicate. (+) percentage represents the induced NO level by IL-1β, and (–) percentage represents the inhibited NO level by AG. The differences of basal level and IL-1β induced NO production between 4 different groups were statistically significant ($p \leq 0.05$).

When chondrocytes from the young and old cartilage were treated with IL-1β, inflammatory cytokine and an inducer of the iNOS gene, NO production was found to decrease with age (Figure 1). Interestingly, the cells from OA-affected cartilage treated with IL-1β did not elevate the already high levels of NO, suggesting that the level of NO in the inflamed chondrocytes was at its maximum.

Aminoguianidine (AG), a competitive inhibitor of iNOS, completely inhibited the NO production in the chondrocytes from young and old cartilages (Figure 1). However, at the same concentration of AG, the production of both basal NO and iNOS was partially inhibited in chondrocytes from OA-affected individuals.

The age-related decline in IL-1-induced NO production by chondrocyte is consistent with the observation that the ability of human chondrocytes to produce NO in response to IL-1β declines with passage (Blanco *et al.*, 1995; Hauselmann *et al.*, 1998). Furthermore, we have recently found that basal NO production is raised with an increasing passage number in young chondrocytes (Figure 2). These results provide some view of an alternative regulation of NO production in articular chondrocytes during ageing and differentiation.

We have found that old chondrocytes from OA-affected cartilage show progressive age-related changes of NO production with markedly decreased inhibition of NO production by AG (1 mM) in comparison with young chondrocytes. In general, AG is a specific inhibitor for iNOS, but incomplete inhibition of the constitutive NO production by AG is observed in OA-affected chondrocytes. A possible explanation may be that there is unexpected NO production by other constitutive isoforms, such as neuronal NOS (ncNOS) or endothelial NOS (eNOS), in OA-affected chondrocytes. The increased expression of OA-NOS, which is similar to ncNOS was demonstrated in OA (Amin *et al.,* 1995).

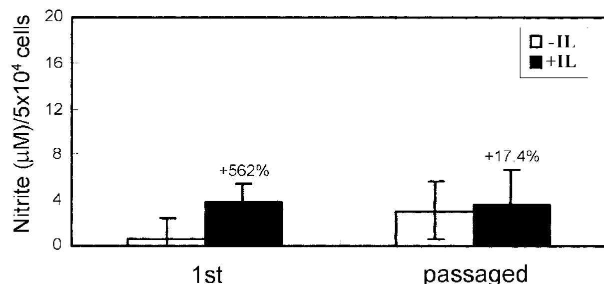

Figure 2. Influence of passage on basal and IL-1-induced NO production by young human articular chondrocytes. Chondrocytes were plated after first passage (1st) or after 5 to 10 passages (passaged). Chondrocytes were cultured for 48 h with culture medium alone, or with IL-1β. Results represent four separate experiments, each performed in duplicate, and are shown as mean±S.D. (+) percentage represents the induced NO level by IL-1β. The difference of basal level and IL-1β induced NO production when comparing the 1st passage to the several passages young chondrocytes was statistically different ($p \leq 0.05$).

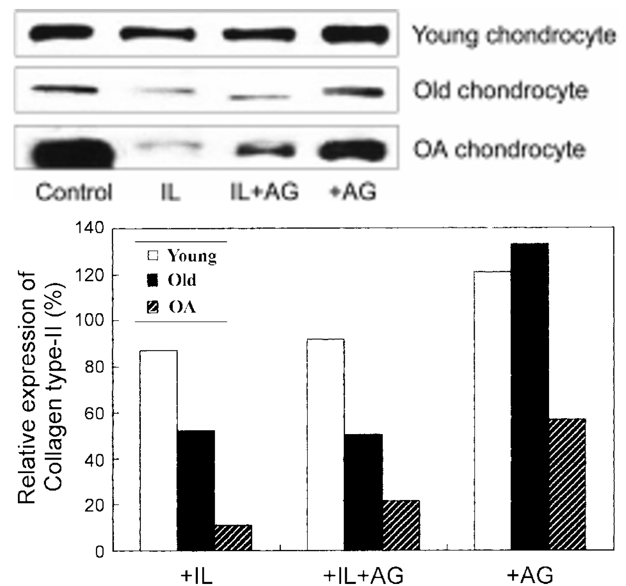

Figure 3. The effect of IL-1β on collagen type II expression by human articular chondrocytes. The articular chondrocytes from young, old and OA-affected cartilage were incubated for 48 h with IL-1β (5 ng/ml), AG (aminoguanidine, 1 mM), either singly or in the indicated combination. Total protein extracted from cells was subjected to SDS-PAGE, and Western blot analysis was carried out for type II collagen as described in Materials and Methods. Densitometric analysis was performed to compare the relative amount of type II collagen.

Even though the precise role of NO in collagen synthesis in chodrocytes has not been reported yet, NO has been known to inhibit the synthesis of type II collagen in the post-translational processing (Cao *et al.*, 1997). In addition, previous studies indicate that the degradation of proteoglycan and collagen was stimulated by metalloproteinase production and activation (Murrel *et al.*, 1995). In our study, different inhibitions of collagen type II production by IL-1β were demonstrated in young and old chondrocytes (Figure 3). In old chondrocytes and OA-affected chondroyctes the collagen type II production was inhibited about 50% by IL-1β, and AG did not overcome this inhibition. However, the inhibition of collagen production by IL-1β was not so prominent in young chondorycytes, although the IL-1β induced NO production was much more than that of old chondroyctes and OA-affected chondrocytes. This result suggest that IL-1β controls collagen homeostasis through more complicated mechanism, at least one of which is dependent on the production of NO. And the complexity of the mechanism probably account for the variable responses of chondrocytes from different ages.

Another possible explanation for our results is an age-dependent change of zonal variations of articular cartilage, which could influence the measured response in mixed cell suspension from entire thickness of the tissue. We have recently developed a method for fractionation of articular chondrocytes from the entire thick-

ness of the tissue, and demonstrated the differences of the responsiveness to IL-1β and the expression of collagen type II between the fractionated cells in rabbit (Min *et al*., 2002). Further studies will be necessary to investigate the differences of fractionated human articular chondrocytes in young, old and OA-affected cartilages.

In conclusion, the results of the present study suggest age-related differences in the regulation of NO production, which may affect the ageing cells and osteoarthritic changes in some way. This study also provides new insights into the topographical pattern of NOS activity through the human articular cartilage. To date, no information is available regarding the differences of NO production between weight bearing and non-weight bearing regions of articular cartilage. We are currently investigating the expression of NO synthase in these types of cells by immunohistochemistry and Western blot analysis.

Acknowledgments

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