

Water-Soluble C60-(OH)₂₄ Fullerene Hydroxide Protects against the Catabolic Stress-Induce Downregulation of Chondrocyte Activity in Osteoarthritis

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Abstract

Recently, attention has been attracted to the roles of oxidative stress and related chondrocyte aging in the pathogenesis of osteoarthritis (OA). To prevent the degeneration of articular cartilage in OA, we focused on water-soluble fullerene hydroxide C60(OH)₂₄, a novel and strong free radical scavenger, as an anti-oxidative agent.

Water-soluble C60(OH)₂₄ was studied for its potential as a protective agent against catabolic stress-induced degeneration of articular cartilage in vitro. In the presence or absence of C60(OH)₂₄ (1.0 nM, 10.0 nM, 100.0 nM), human chondrocytes were incubated with IL-1 β (1.0 ng/ml). After the 24-hour incubation period, chondrocyte activity was analyzed.

Results indicated that C60(OH)₂₄ inhibited the OA-relating catabolic responses (IL-1 β -induced production of cartilage degrading enzyme, IL-1 β -induced down regulation of proteoglycan production and glucose metabolism) in OA chondrocytes. C60(OH)₂₄ also reduced the IL-1 β -induced upregulation DNA repair enzyme, apurinic/aprimidinic endonuclease 2 (Apex 2), in OA chondrocytes.

Our findings suggest that C60(OH)₂₄ may have a potential to protect against the IL-1 β -induced downregulation of chondrocyte activity in OA. These data provide a novel pathogenic mechanism linking oxidant-mediated DNA damage and OA chondrocytes.

Keywords: Osteoarthritis; C60 fullerene; DNA repair enzyme; Oxidative stress; Chondrocytes

Abbreviation: OA: Osteoarthritis; ROS: Reactive oxygen species; Apex2: Apurinic/aprimidinic endonuclease; PBS: Phosphate-buffered saline; DMEM: Dulbecco's modified Eagle's medium; HEPES: 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid; IL: Interleukin β .

Introduction

Mechanical and chemical stresses to articular cartilage influence the stable cellular activities of chondrocytes and stimulate the production of proinflammatory cytokines and matrix protein [1,2]. There is a general consensus that mechanical stressed chondrocytes produce large amounts of reactive oxygen species (ROS: superoxide, nitric oxide, hydrogen peroxide, and peroxyxynitrite) in addition to proinflammatory cytokines and chemokines [3-5]. Several reports demonstrat-

ed that mechanical stress-induced production of ROS could cause direct damage to chondrocytes and cartilage matrix, resulting in the degeneration of articular cartilage [6-10].

Martin et al. demonstrated that aging decreases the ability of chondrocytes to maintain and repair articular cartilage [11-13]. They measured three different biomarkers for cellular aging in articular cartilage from human donors and found that aging of chondrocytes in vivo correlates with the

age of the donor. Their findings suggest that the association between OA and aging is due, at least in part, to age-related loss of chondrocyte and deterioration of matrix functions. It is thought that aged chondrocytes show an insufficient response to anabolic factors, which results in continuous matrix degradation from unbalanced catabolic and anabolic activities [11,14]. Recent reports revealed that chondrocyte aging is closely involved in oxidative stress in the degenerated articular cartilage [11-14]. Moreover, recent studies showed that oxygen free radicals directly damage the guanine repeats in the telomere DNA [15,16]. This may result in earlier cellular senescence. The evidence for chondrocyte aging caused by ROS in OA cartilage suggests that oxidative stress is implicated in the development of OA.

We demonstrated in a model of OA in vitro and in vivo that fullerene (C60), a strong free-radical scavenger, can function as a protective agent against IL- β induced degeneration of articular cartilage. C60 is a molecule composed completely of carbon in a unique spherical structure that can act as a strong radical sponge [14,15]. This compound has a high reactivity with oxygen free radicals and can potentially act as a free radical scavenger. It has been reported that the antioxidant level of C60 is several hundred-fold higher than that of other antioxidants [14,17,18]. Recently, C60 fullerene has shown to inhibit neuronal apoptosis by scavenging oxygen reactive species [19] and protects human skin keratinocytes from ROS-induced cell death after UV stress [20]. This suggests that C60 is a useful agent to protect against the oxygen free radical-induced pathological features in a variety of diseases [21]. Use of fullerene could lead to the development of novel therapeutic strategies in the prevention of both chondrocyte aging and cartilage degeneration. We have previously confirmed that μM order level of C60 (10~100 μM) inhibits the IL- β induced production of cartilage matrix degrading enzyme (matrix metalloproteinase-3, MMP-3) and the down-regulated production of cartilage matrix proteins (type II collagen and proteoglycan), cellular senescence and apoptosis in chondrocytes in vitro [14]. In our previous study, since C60 is water insoluble, we used cyclodextrin clathrate C60 fullerene.

In this study, we studied the therapeutic effects of water soluble C60(OH)₂₄ on chondrocyte activity and energy metabolism, which were monitored by the production of articular cartilage component (proteoglycan) and the uptake of glucose in the cells, respectively. There is a general consensus that osteoarthritic chondrocytes produce a lower level of proteoglycan and a higher level of cartilage-degrading enzyme MMP-3 in comparison with normal chondrocytes (3-5). More recently, we demonstrated that the DNA repair enzyme, apurinic/apyrimidinic endonuclease (Apex) 2, is involved in the degeneration of articular cartilage on OA [22]. Our previous study revealed that expression of DNA repair enzyme Apex2 in chondrocytes was associated with the degeneration of articular cartilages and was induced by OA-relating catabolic factors [22]. Thus, we studied effects of C60(OH)₂₄ on chondrocyte activities (production of proteoglycan and MMP-3), energy metabolism (glucose uptake) and the expression of Apex 2 in osteoarthritic chondrocytes.

Our previous study revealed that cyclodextrin clathrate C60 showed inhibitory effects on OA relating catabolic responses with the concentration of μM order [14]. In contrast, we here demonstrate that C60(OH)₂₄ inhibits the OA-relating catabolic responses (IL-1 β -induced production of cartilage degrading enzyme, IL-1 β -induced downregulation of proteoglycan production and glucose metabolism) of OA chondrocytes in the order of 1/1000 concentration in comparison with cyclodextrin clathrate C60. Furthermore, we demonstrate that C60(OH)₂₄ reduces the IL- β induced upregulation of DNA oxidative damage and expression of Apex 2 in OA chondrocytes. Our findings suggest that C60(OH)₂₄ may have a potential to protect against the catabolic factor-induced downregulation of chondrocyte activity in OA. These data provide a novel pathogenic mechanism linking oxidant-mediated DNA damage and OA chondrocytes.

Materials and Methods

Our previous study revealed that cyclodextrin clathrate C60 showed inhibitory effects on OA relating catabolic responses with the concentration of μM order [14]. In contrast, we here demonstrate that C60(OH)₂₄ inhibits the OA-relating catabolic responses (IL-1 β -induced production of cartilage degrading enzyme, IL-1 β -induced downregulation of proteoglycan production and glucose metabolism) of OA chondrocytes in the order of 1/1000 concentration in comparison with cyclodextrin clathrate C60. Furthermore, we demonstrate that C60(OH)₂₄ reduces the IL- β induced upregulation of DNA oxidative damage and expression of Apex 2 in OA chondrocytes. Our findings suggest that C60(OH)₂₄ may have a potential to protect against the catabolic factor-induced downregulation of chondrocyte activity in OA. These data provide a novel pathogenic mechanism linking oxidant-mediated DNA damage and OA chondrocytes.

Chondrocyte isolation and cell culture

Human articular cartilage samples were obtained from knee joints during arthroplastic joint surgery for OA ($n=9$; mean age-73.6 years (range, 63–81 years)) after obtaining informed consent from the patients. The protocol of this study was accepted in our University ethical committee in St. Marianna University School of Medicine. The cartilage explants were cut into small pieces, washed with PBS and digested with 1.5 mg/ml collagenase B (Sigma, St. Louis, MO, USA) in Dulbecco's modified Eagle's medium (DMEM) (Sigma) at 37 °C overnight on a shaking platform. The isolated chondrocytes were centrifuged, washed three times with PBS, resuspended and cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 25 mM HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid), and 100 U/ml penicillin and streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO₂.

Production of proteoglycan and MMP-3

Cultured chondrocytes were seeded on 10 cm culture dish (Becton, Dickinson and Company, New Jersey) at about 2.0×10^5 cells per dish and incubated with DMEM plus 10% FCS in the presence or absence of IL-1 β (1 ng/ml) or

C60(OH)₂₄ (1.0, 10.0, or 100.0 nM) for 24 hours at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. After the incubation period, the supernatant (culture conditioned medium) was collected from each dish. C60(OH)₂₄ was purchased from VitaminC60 Bio (Tokyo, Japan).

To examine the effect of C60(OH)₂₄ on anabolic activity in chondrocytes, the levels of proteoglycan produced by chondrocytes were measured using an enzyme-linked immunosorbent assay (ELISA) kit in chondrocyte culture medium in accordance with the manufacturer's protocol (DIA source Immuno Assays S.A., Nivelles, Belgium). For measuring the catabolic activity of chondrocytes, the level of MMP-3 produced by chondrocytes was measured using an ELISA kit (R&D Systems Inc., Minneapolis, MN). Data from four independent experiments were analyzed.

Measurement of glucose metabolism in chondrocytes

Cultured chondrocytes were seeded on 10 cm culture dish (Becton, Dickinson and Company, New Jersey) at about 1.3×10^6 cells per dish and incubated with DMEM plus 10% FCS in the presence or absence of IL-1 β (1 ng/ml) or C60(OH)₂₄ (1.0, 10.0, or 100.0 nM) for 24 hours at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. After the incubation period, cultured chondrocytes were subsequently processed for glucose metabolism assay as following.

To examine the effect of C60(OH)₂₄ on cellular metabolic activity in chondrocytes, the levels of glucose metabolism in chondrocytes were measured using non-radioisotopic enzymatic photometric assay for measurement of 2-deoxyglucose (2DG) uptake in chondrocyte cell cultures in accordance with the manufacturer's protocol (COSMO BIO co ltd, JAPAN). Measurement of 2DG uptake in cultured cells is a reliable approach with which to estimate glucose uptake and thereby to explore the regulation of glucose metabolism of cells.

Immunocytochemistry for Apex 2 in OA chondrocytes

Cultured chondrocytes were seeded on 8-well glass chamber slide (BD BioCoat, Two Oak Park Bedford, MA USA) at about 2×10^4 cells per well and incubated with DMEM in the presence or absence of IL-1 β (1.0 ng/ml) or C60(OH)₂₄ (1.0, 10.0, 100.0 nM) for 24 hours. Chamber slides were subsequently processed for immunocytochemical staining.

For immunostaining for Apex 2 in chondrocytes, cells on chamber slides were fixed with 2% parahormaldehyde and immunostained with antibodies recognizing Apex2. Chondrocytes on chamber slides were treated with 3% H₂O₂, followed by blocking of nonspecific protein binding with a blocking agent (Protein Block, Dako, Carpinteria, CA, USA). The cells were then incubated with monoclonal antibodies against Apex 2 (1:200 dilution; Abcam Inc., Cambridge, UK) for 1 h at room temperature, followed by incubation with biotinylated goat anti-mouse IgG (Dako) for 30 min at room temperature. Af-

ter washing with phosphate-buffered saline (PBS), the sections were incubated with a streptavidin-horseradish peroxidase complex (LSAB2 Kit; Dako) for 30 min at room temperature. Diaminobenzidine (Sigma) was used as a visible peroxidase reaction product, and the sections were counterstained with Mayer's haematoxylin (Sigma). The number of Apex2-positive cells was counted in five degenerated articular cartilage areas under high-power magnification ($\times 400$) for each case, and the mean number of positive cells per high-power field was calculated. Three independent observers assessed cartilage damage in a blinded manner.

Statistical analysis

The results for each experimental condition were determined from the mean of triplicate trials. Data were expressed as means \pm standard deviation. A two-tailed Student's *t*-test was used to assess the significance of differences between two groups. Analysis of variance was used for comparisons of more than two groups, and differences between two groups within the set were analysed by a Fisher's protected least-significant difference test. Probability values of <0.05 were considered significant.

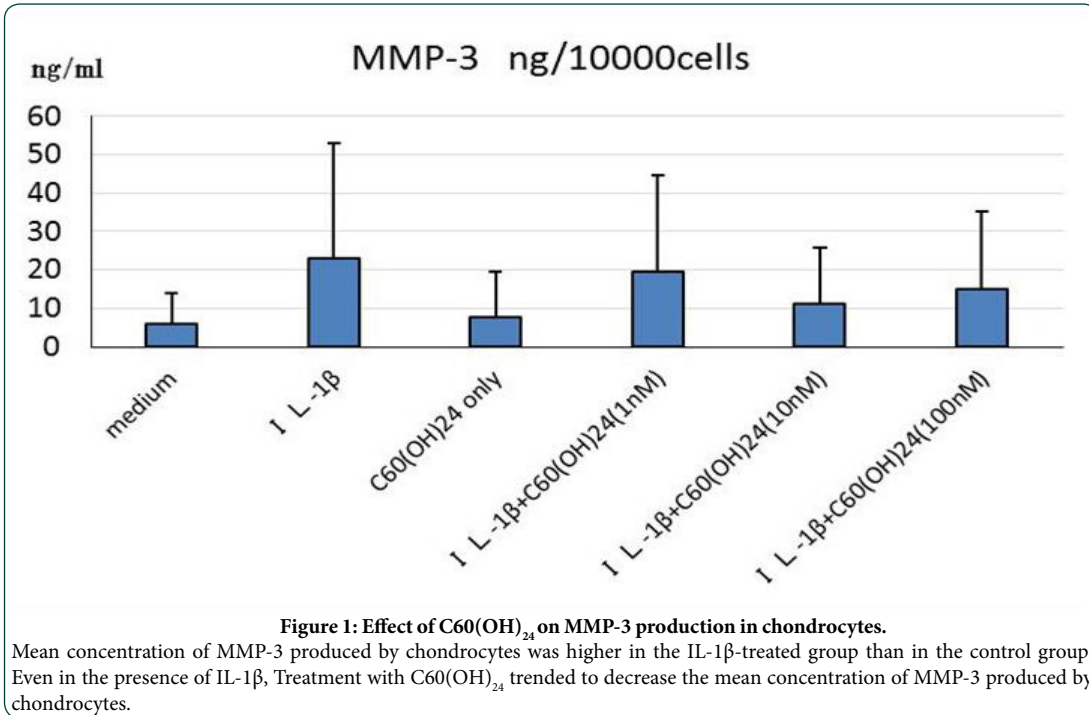
Results and Discussion

Inhibitory effects of C60(OH)₂₄ on the IL- β induced production of cartilage matrix degrading enzymes, MMP-3 in chondrocytes

As shown in Figure 1, mean concentration of MMP-3 production from human osteoarthritic chondrocytes was higher in the IL- β -treated group (IL-1 β : 1.0 ng/ml) than in the control (medium only). Although there was no significant difference between the control and the IL-1 β -treated group, IL-1 β stimulated the MMP-3 production from chondrocytes. IL-1 β is well known to be the OA-relating catabolic cytokine that is closely involved in the pathogenesis of OA.

Interestingly, in comparison with the MMP-3 (ng/10000 cells) production from the IL-1 β -treated chondrocytes, there was a tendency to decrease mean concentrations of MMP-3 from chondrocytes that were treated with C60(OH)₂₄ (1.0, 10.0, or 100.0 nM) even in the presence of IL-1 β (1.0 ng/ml). These findings suggest that C60(OH)₂₄ may have a potential to inhibit the IL-1 β -stimulated secretion of MMP-3 in OA chondrocytes.

Our previous study demonstrated that MMPs productions from chondrocytes were inhibited at a μ M order of cyclodextrin clathrate C60 fullerene (C60-cyclodexytrin) [14]. In the current study, we found that even nM order of C60(OH)₂₄ showed the tendency to inhibit the catabolic factor-stimulated production of MMP-3 in OA chondrocytes. Regarding the production of MMP-3 from chondrocytes, the effective concentration of C60(OH)₂₄ was 1000-fold lower than that of C60-cyclodexytrin. The difference in the effective concentration between C60(OH)₂₄ and C60-cyclodexytrin may be due to the different degree of their water-solubility.

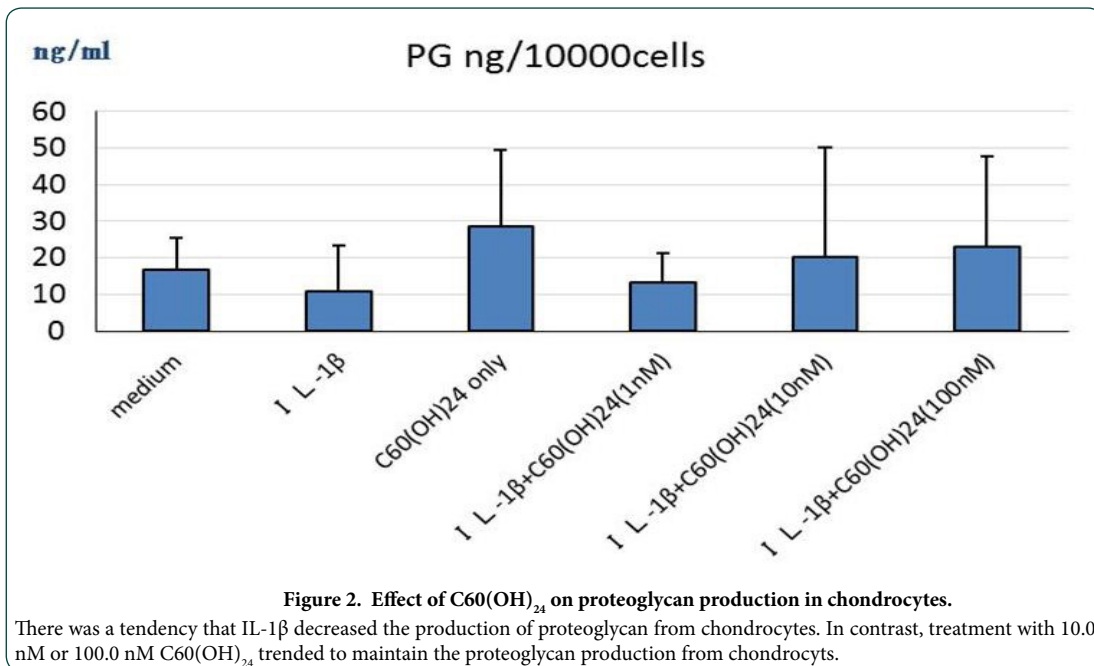


Effect of C60(OH)₂₄ on production of articular proteoglycan from chondrocytes

Figure 2 showed levels of productions of articular cartilage matrix component, proteoglycan, from chondrocytes in the presence or absence of IL-1β or C60(OH)₂₄. IL-1β(1.0 ng/ml) decreased the production of proteoglycan from chondrocytes, although there was no significant difference between the control group (medium only) and the IL-1β-treated group.

cytes. The maintenance potential showed 1000-fold lower in C60(OH)₂₄ (nM order) than in C60-cyclodextrin (μM order) that was analyzed in our previous study [14].

Interestingly, treatment with only C60(OH)₂₄ also accelerated the proteoglycan secretion from chondrocytes in comparison with the control (medium only). This suggests that C60(OH)₂₄ may stimulate proteoglycan production from chondrocytes via the elimination of cellular respiration-pro-



In contrast, even in the presence of IL-1β, treatment with C60(OH)₂₄ trended to maintain the level of proteoglycan production from chondrocytes. These findings indicate that C60(OH)₂₄ has a potential to protect against the IL-1β-induced down-regulation of proteoglycan production in OA chondro-

duced ROS by C60 fullerene as an anti-oxidant. Oxygen free radical spontaneously generated during the cellular respiration may directly damage DNA and gene as an oxidative stress. In normal conditions, cellular antioxidants eliminate the ROS to protect against oxidative stress and maintain cellular activity.

However, in pathologic conditions, excess generation of ROS and depletion of cellular antioxidants may induce oxidative damage in the cells. As an antioxidant, C60(OH)₂₄ may aid by supplementing the cell's own antioxidants in the elimination of excess ROS in the maintenance of cellular activity.

Effect of C60(OH)₂₄ on glucose metabolism in chondrocytes

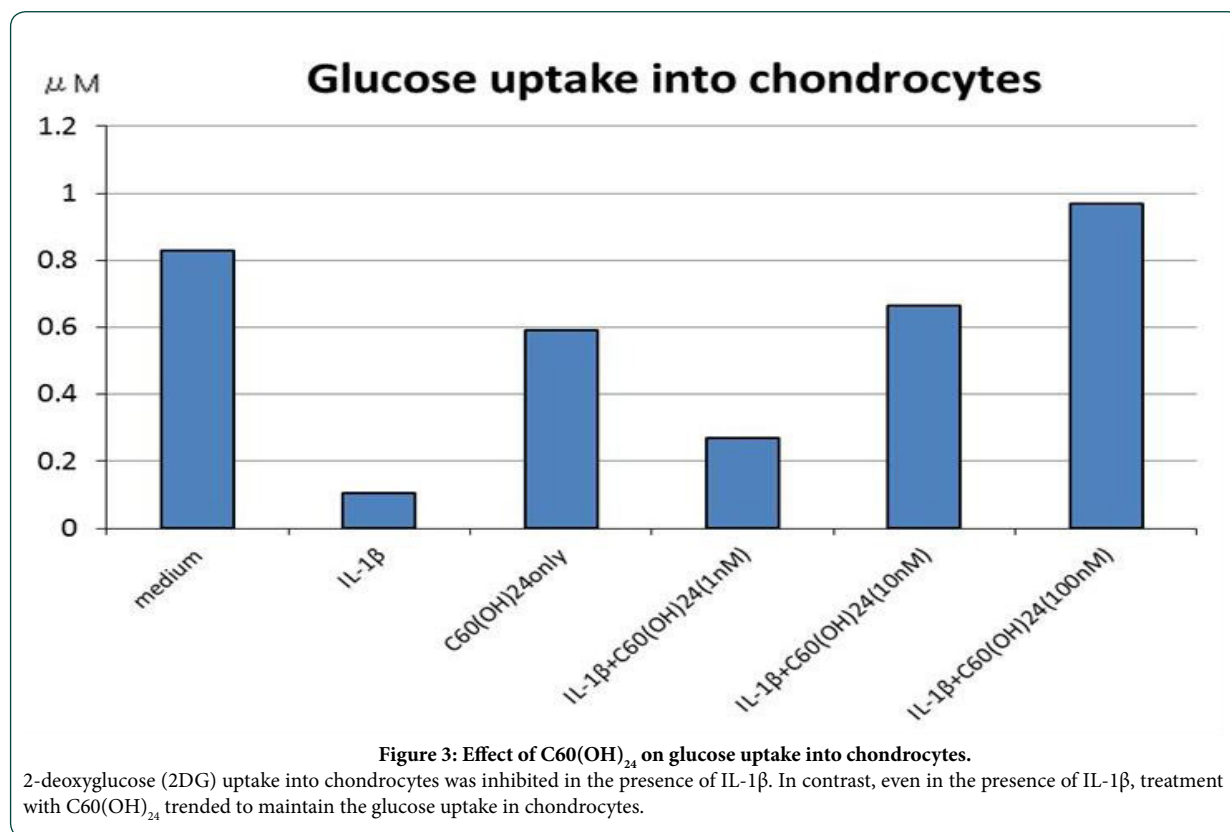
We examined the effect of C60(OH)₂₄ on chondrocyte glucose metabolism. The levels of glucose metabolism in chondrocytes were analyzed by the glucose uptake into the cells.

In Figure 3, there was a tendency to decrease the 2DG uptake into chondrocytes by treatment of IL-1 β (1.0 ng/ml), suggesting that OA relating factor, IL-1 β , may inhibit the glucose metabolism of chondrocytes. In contrast, even in the presence of IL-1 β , Treatment with C60(OH)₂₄ maintained the uptake of 2DG in chondrocytes. These findings indicate that C60(OH)₂₄ could protect against the IL-1 β -induced down-regulation of glucose metabolism in OA chondrocytes.

age [24]. We have postulated that the change of cellular anti-oxidative activity through the Apex 2-associated DNA repair pathway in degenerated articular cartilage may, at least in part, participate in the development of OA cartilage degeneration. Therefore, in the present study, we examined whether or not C60(OH)₂₄ influenced the expression of Apex 2 in chondrocytes.

To clarify the effect of C60(OH)₂₄ on the Apex 2 expression in chondrocytes, the immunopositivities for Apex 2 were analysed in cultured chondrocytes that were incubated in the presence or absence of IL-1 β or C60(OH)₂₄. Figure 4 shows representative images of immunohistochemical staining for Apex 2 (chondrocytes isolated from 81 year-old female patients with OA).

As shown in Figure 4, a fine granular pattern of Apex 2 was detected in cultured OA chondrocytes. A higher expression pattern of Apex 2 was observed in the IL-1 β -treated chondrocytes. This finding suggests that OA relating catabolic fac-



Effect of C60(OH)₂₄ on DNA repair enzyme Apex 2 in chondrocytes

To counteract oxidative damage to nucleic acids, human are equipped with several distinct enzymes. One of these, apurinic/aprimidinic (AP) endonuclease 2 (Apex 2) is now thought to have an important role in the protection against DNA oxidative damage [23]. AP sites occur frequently in DNA molecules by spontaneous hydrolysis, by DNA damaging agents or by DNA glycosylases that remove specific abnormal bases [23,24]. More recently, it has been clearly revealed that the base excision repair pathway by Apex2 is largely responsible for the repair of oxidative stress-induced DNA dam-

tor, IL-1 β , may induce catabolic reactions in OA chondrocytes and their catabolic reactions including DNA oxidative damage in the cells may require DNA repair enzymes, such as Apex 2. In contrast, the chondrocytes were stained more weakly in the IL-1 β + C60(OH)₂₄-treated chondrocytes [C60(OH)₂₄: 10.0 nM, 100.0 nM] compared with the IL-1 β -treated chondrocytes.

Immunopositivity for Apex 2 was statistically predominant in the IL-1 β -treated chondrocytes in comparison with the control chondrocytes in both patients (Figure 5AB, chondrocytes from 70 year-old patient: P=0.045, 81 year-old

patient: $P=0.047$).

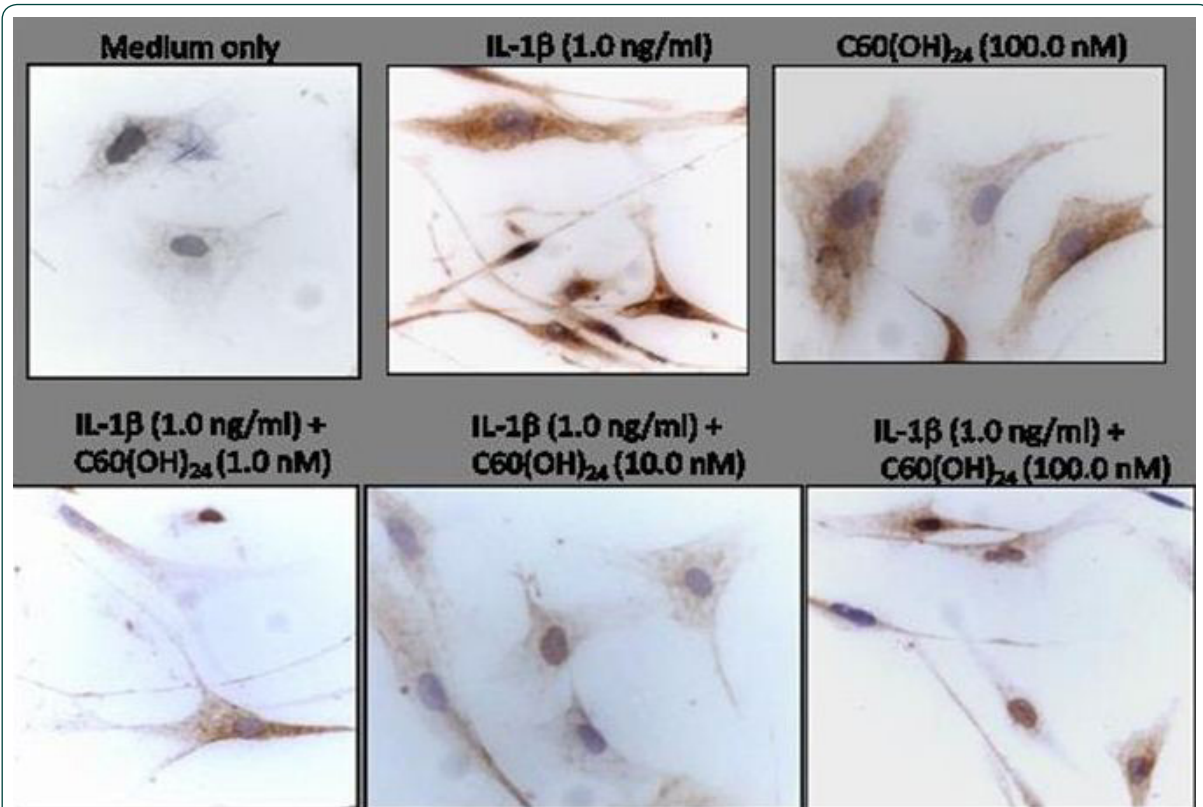


Figure 4. Representative images of immunohistochemical staining for Apex 2 in chondrocytes.

Treatment with IL-1 β induced the expression of Apex 2 in chondrocytes. Even in the presence of IL-1 β , C60(OH) $_{24}$ reduced the overexpression of Apex 2 in chondrocytes.

In 70 year-old patient with OA, treatment with C60(OH) $_{24}$ trended to inhibit the IL-1 β -induced overexpression of Apex 2 in chondrocytes, although there were no significant differences between the IL-1 β group and C60(OH) $_{24}$ treated groups [Figure 5A, IL-1 β vs. 1.0 nM C60(OH) $_{24}$: $P=0.102$,

1.0 nM C60(OH) $_{24}$: $P=0.169$, IL-1 β vs. 10.0 nM C60(OH) $_{24}$: $P=0.006$, IL-1 β vs. 100.0 nM C60(OH) $_{24}$: $P=0.007$]. These findings indicate that C60(OH) $_{24}$ may work as an anti-oxidant and decrease the IL-1 β -mediated excess production of ROS in chondrocytes. Therefore, overexpression of Apex 2 may be not

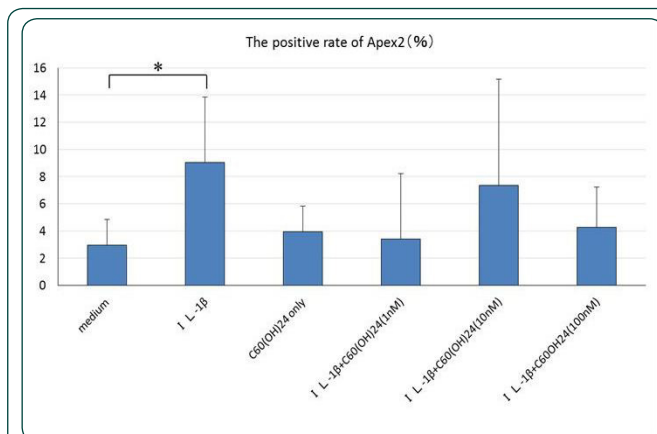


Figure 5 A

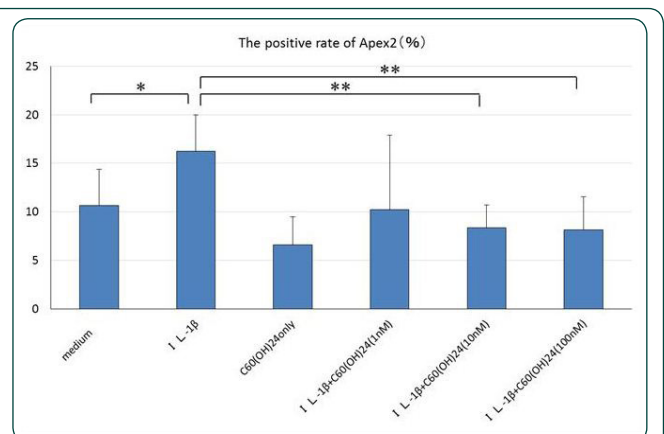


Figure 5 B

Figure 5: Immunopositivity for Apex 2 in donor chondrocytes.

Immunopositivity for Apex 2 in IL-1 β -treated group was significantly higher than in the control. C60(OH) $_{24}$ -treated groups trended to decrease the IL-1 β -induced expression of Apex 2 in chondrocytes. (* $P<0.05$, ** $P<0.01$)

A. Immunopositivity for Apex 2 in chondrocytes from 70 years old, female.

B. Immunopositivity for Apex 2 in chondrocytes from 81 years old, female.

IL-1 β vs. 10.0 nM C60(OH) $_{24}$: $P=0.698$, IL-1 β vs. 100.0 nM C60(OH) $_{24}$: $P=0.105$]. In 81 year-old patient with OA, treatment with 10.0 nM and 100.0 nM C60(OH) $_{24}$, but not 1.0 nM C60(OH) $_{24}$, significantly inhibited the IL-1 β -accelerated overexpression of Apex 2 in chondrocytes [Figure 5B, IL-1 β vs.

required even in the presence of OA-related catabolic factor, IL-1 β . Although further studies are needed to clarify the exact mechanism of Apex 2 expression and the role of C60(OH) $_{24}$ in osteoarthritic chondrocytes, C60(OH) $_{24}$ may be able to play a role in the protection against catabolic processes (secretion of

cartilage matrix degrading enzyme; down-regulation of proteoglycan production, glucose metabolism, and DNA repair enzyme Apex 2 in chondrocytes) in the development of OA.

Conclusion

In our study, we isolated human cultured chondrocytes from 10 patients with osteoarthritis. Each experiment was triplicated using chondrocytes from 10 different patients with OA. Indeed, in some experiments, considerable variation of data may be due to variability of cellular activities in chondrocytes that were isolated from patients with different disease activities. Although further studies are needed to clarify the statistically exact effects of C60(OH)₂₄ on chondrocyte activities in OA, the study reported here sought to demonstrate in the OA model in vitro that water soluble C60(OH)₂₄ can function as a protective agent against the catabolic stress-induced degeneration of articular cartilage.

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