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The *in vitro* effects of dehydroepiandrosterone on human osteoarthritic chondrocytes

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Summary

Objective: To investigate the *in vitro* effects of dehydroepiandrosterone (DHEA) on human osteoarthritic chondrocytes.

Design: Chondrocytes isolated from human osteoarthritic knee cartilage were three-dimensionally cultured in alginate beads, except for cell proliferation experiment. Cells were treated with DHEA in the presence or absence of IL-1β. The effects on chondrocytes were analyzed using a 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay (for chondrocyte proliferation), a dimethylmethylene blue (DMB) assay (for glycosaminoglycan (GAG) synthesis), and an indole assay (for DNA amount). Gene expressions of type I and II collagen, metalloproteinase-1 and -3 (MMP-1 and -3), and tissue inhibitor of metalloproteinase-1 (TIMP-1) as well as the IL-1β-induced gene expressions of MMP-1 and -3 were analyzed by reverse transcription-polymerase chain reaction (RT-PCR). The protein synthesis of MMP-1 and -3 and TIMP-1 was determined by Western blotting.

Results: The treatment of chondrocytes with DHEA did not affect chondrocyte proliferation or GAG synthesis up to 100 μM of concentration. The gene expression of type II collagen increased in a dose-dependent manner, while that of type I decreased. DHEA suppressed the expression of MMP-1 significantly at concentrations exceeding 50 μM. The gene expression of MMP-3 was also suppressed, but this was without statistical significance. The expression of TIMP-1 was significantly increased by DHEA at concentrations exceeding 10 μM. The effects of DHEA on the gene expressions of MMP-1 and -3 were more prominent in the presence of IL-1β, in which DHEA suppressed not only MMP-1, but also MMP-3 at the lower concentrations, 10 and 50 μM, respectively. Western blotting results were in agreement with RT-PCR, which indicates that DHEA acts at the gene transcription level.

Conclusions: Our study demonstrates that DHEA has no toxic effect on chondrocytes up to 100 μM of concentration and has an ability to modulate the imbalance between MMPs and TIMP-1 during OA at the transcription level, which suggest that it has a protective role against articular cartilage loss.
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Key words: Dehydroepiandrosterone, Osteoarthritis, Chondrocyte, Metalloproteinase, Tissue inhibitor of metalloproteinase-1.

Introduction

Osteoarthritis (OA) is a degenerative joint disease characterized by progressive loss of articular cartilage, subchondral bone remodeling, spur formation, and synovial inflammation. OA is believed to be a consequence of mechanical and biochemical events that result in an imbalance between the synthesis and degradation of articular cartilage matrix[1]. The target of therapy is becoming more focused on the specific steps of OA pathophysiology rather than on the broad inhibition of inflammation or the relief of pain. In OA, the principal cause of joint morbidity results from the degradation of the articular extracellular matrix (ECM) of articular cartilage, which results from the activation of various proteases and proinflammatory cytokines. The proinflammatory cytokines are believed to play a pivotal role in the initiation and development of the OA; of those, IL-1β and TNF-α appear prominent[2,3]. IL-1β and TNF-α appear to be first produced by the synovial membrane and then to diffuse into articular cartilage through the synovial fluid. They then activate chondrocytes, which in turn produce many catabolic factors.

Il-1β has been implicated in the transcriptional upregulation of various MMPs, including MMP-1[4,5]and MMP-3[6,7]. The MMPs are an enzyme superfamily of at least 21 members, which can be classified into subgroups of collagenases (MMP-1, -8, -13), stromelysins (MMP-3, -10, -11), gelatinases (MMP-2, -9), and as membrane-type 1 (MMP-14)[8]. The collagenases are distinguished from the other MMPs by their ability to cleave triple helical regions of cartilage type II collagen. Stromelysin-1 (MMP-3) degrades several ECM molecules, including aggrecan and type II collagen[9–11]. In addition, MMP-3 is known to be essential for the full activation of proMMP-1[12].

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The activity of MMPs is controlled by the tissue inhibitor of metalloproteinase (TIMP), a glycoprotein that inhibits all MMPs at a stoichiometry of 1:1, by forming high-affinity complexes[13]. Imbalance between MMPs and TIMPs is well known to be of importance in the progression of OA[14,15].

Dehydroepiandrosterone (DHEA) is a 19-carbon steroid hormone, classified as an adrenal androgen. DHEA is synthesized from pregnenolone (derived from cholesterol) and is rapidly sulfated to its ester form, DHEA-S, the predominant form in circulating plasma[16]. Because of its decline with age, DHEA is well known as an 'antidote for aging', and a number of studies are currently being performed on its role in atherosclerosis[17], cancer[18], diabetes, obesity[19], and aging[20], as well as inflammatory arthritis, such as rheumatoid arthritis[21–23]. In rheumatoid arthritis patients, the serum level of DHEA has been found to be lower than in healthy controls[24], while the cause remains unknown. In addition, the exogenous administration of DHEA was found to offer protection against the development of collagen-induced arthritis in an animal model[25].

While a previous study has reported on the effect of DHEA on collagenase and gelatinase production[26], nothing is known about the effects of DHEA on OA, as far as we are aware. In the present study, we investigated the *in vitro* effects of DHEA on osteoarthritic chondrocytes, including their proliferation, GAG synthesis, and expressions of MMP-1, -3, and TIMP-1. In addition, we assessed the effects of DHEA on the gene expressions of MMP-1 and -3 in the presence of exogenous IL-1β.

Materials and methods

ISOLATION AND EXPANSION OF HUMAN OA CHONDROCYTES

Human OA cartilage was obtained from the femoral condyles of OA patients undergoing knee joint replacement surgery. Cartilage was washed in calcium- and magnesium-free phosphate buffered saline (DPBS) and finely minced. Chondrocytes were released from articular cartilage after being digested for 1 h with 0.2% pronase (Sigma, St. Louis, MO, USA), followed by digestion for 3 h with 0.2% collagenase (Sigma) at 37°C in high-glucose Dulbecco's modified Eagle medium (DMEM; Life Technologies, Rockville, MD, USA) containing antibiotic–antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B; Life Technologies). After removing undigested cartilage using a 70 μm nylon sieve, the chondrocytes were collected by centrifugation, washed twice, resuspended in DMEM supplemented with 10% fetal bovine serum (FBS; Life Technologies), 25 μg/ml L-ascorbic acid (Sigma) and antibiotics (hereafter called growth medium), and finally plated in 100-mm tissue culture dish for expansion at 37°C in a humidified atmosphere of 5% CO_2 for 7 days.

CULTURE OF CHONDROCYTES IN ALGINATE BEADS

After 7 days in monolayer culture, chondrocytes were washed twice and harvested with trypsin–EDTA (0.25% trypsin, 0.53 mM EDTA; Life Technologies). They were then centrifuged, washed twice, and suspended in a 1.2% solution of sterile alginate in 0.15 M NaCl at a density of 4×10^6 per ml. The cell suspension was slowly expressed through a 22-gauge needle, dropped into a 102 mM $CaCl_2$ solution, and allowed to polymerize for 10 min. Beads were washed twice with 0.15 M NaCl, twice in DMEM, and were then transferred to six-well plate and cultured for 5 days before experiments in growth medium at 37°C in a humidified atmosphere of 5% CO_2.

TREATMENT OF DHEA AND RECOMBINANT HUMAN IL-1β

In a series of experiments on cell proliferation and GAG synthesis, chondrocytes in monolayer culture for proliferation assay or chondrocytes in alginate beads for GAG assay were incubated in growth medium with 0, 10, 50, or 100 μM of DHEA (Sigma) for 7 days. In another series of experiments on the gene expressions of MMP-1, -3, and TIMP-1, alginate beads were incubated in growth medium with 0, 10, 50, or 100 μM of DHEA for 3 days. In addition, to examine the effects of DHEA on IL-1β-induced gene expressions of MMP-1 and -3, beads were incubated in growth medium with 0, 10, 50, or 100 μM of DHEA in the presence of 1000 pg/ml of recombinant human IL-1β (IL-1β; Calbiochem, CA, USA) for 3 days. For Western blot analysis, beads were incubated in growth medium with 0, 10, and 100 μM of DHEA for 3 days. The growth medium and IL-1β were changed every other day.

CELL PROLIFERATION ASSAY

Chondrocytes proliferation assay was performed using the 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) method using a commercially available kit (CellTiter 96 Aqueous; Promega, Madison, WI, USA) according to the manufacturer's instruction. Briefly, after 7 days in monolayer culture, chondrocytes were washed twice and harvested with trypsin–EDTA (0.25% trypsin, 0.53 mM EDTA; Life Technologies). The chondrocytes were centrifuged, washed twice, and resuspended in growth medium at a density of 5×10^3 cells per well in a 96 multiwell plate. At designated times (1, 3, and 7 days), 100 μl of phenazine methosulfate (PMS) solution was added to 2.0 ml of MTS solution, immediately before the mixture was added to the cells. The cells were incubated for 90 min at 37°C in a humidified atmosphere of 5% CO_2. Plates were read on a microplate spectrophotometer ($Versa_{max}$; Molecular Devices Corp., Sunnyvale, CA, USA) at 492 nm.

GLYCOSAMINOGLYCAN AND DNA AMOUNT ASSAY

For glycosaminoglycan (GAG) assay, the media were harvested every 3 days and pooled for each 7-day period. At each specified time (1, 3, and 7 days), the beads were dissolved as described previously and centrifuged at 300×*g* for 10 min at 4°C, obtaining two fractions: a supernatant containing macromolecules from the further-removed compartment; and a pellet containing cells with their cell-associated matrix (CM). The media, supernatant, and pellet were digested for 12 h at 55°C in papain buffer (200 μg/ml papain in 50 mM EDTA, 5 mM L-cystein, pH 3.0). GAG amount was quantified using dimethylmethylene blue (DMB) assay[27]. The metachromatic reaction of GAG with DMB was monitored using a spectrophotometer, and the ratio A_{540}:A_{595} was used to determine the amount of GAG present, using chondroitin sulfate C (Sigma) as a standard. The amount of DNA in each sample collected by centrifuging of the papain-treated pellet was determined using indole assay[28]. Total amount of GAG was normalized vs the total amount of DNA.

Table I
Polymerase chain reaction primer sequences

Gene	Sequences	Genebank accession number
GAPDH		BC014085
Sense	5′-ATTGTTGCCATCAATGACCC-3′	
Antisense	5′-AGTAGAGGCAGGGATGATGTT-3′	
Type I collagen		S64596
Sense	5′-CTCGAGGTGGACACCACCCT-3′	
Antisense	5′-CAGCTGCATGGCCACATCGG-3′	
Type II collagen		NM_033150
Sense	5′-GAATTCGGTGTGGACATAGG-3′	
Antisense	5′-TACAGAGGTGTTTGACACAG-3′	
MMP-1		NM_002421
Sense	5′-ATTCTACTGATATCGGGGCTTTGA-3′	
Antisense	5′-ATGTCCTTGGGGTATCCGTGTAG-3′	
MMP-3		AF405705
Sense	5′-CTCACAGACCTGACTCGGTT-3′	
Antisense	5′-CACGCCTGAAGGAAGAGATG-3′	
TIMP-1		BC007097
Sense	5′-AATTCCGACCTCGTCATCAGG-3′	
Antisense	5′-ACTGGAAGCCCTTTTCAGAGC-3′	

REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

Total RNA was extracted using a RNA extraction kit (Qiagen, Germany). The cDNA was synthesized using 0.5 μg of RNA and random hexamers in a commercially available kit (First Strand cDNA Synthesis kit; MBI Fermentas, Lithuania) according to the manufacturer's recommendations. The resulting cDNA was then amplified by PCR using another commercially available kit (Accupower; Bioneer, South Korea) in 20 μl using three thermocycler temperatures (Perkin–Elmer, Norwalk, CT, USA). Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) reverse transcriptase-polymerase chain reaction (RT-PCR) products were used for normalization. All reactions were determined to be in a linear range of amplification within cyclic numbers 14 to 30. Primers used for human GAPDH, type I and II collagen, MMP-1 and -3, and TIMP-1 are itemized in Table I. A single cycle consisted of 30 s denaturation at 94°C, 30 s annealing at 60°C, and 30 s extension at 72°C. A 10 μl sample of the reaction mixture was electrophoresed on 1.5% agarose gel containing ethidium bromide to evaluate the amplification and determine the size of the generated fragments. A 100 bp DNA ladder (Bioneer) was used as a standard size marker. A densitometric computer program (TINA; Raytest Isotopenmeßgerate, Germany) was used to analyze the scan of the RT-PCR agarose gel after photographic documentation. The program measured the relative mean density of the bands of the PCR products corrected with respect to the background.

WESTERN BLOT ANALYSIS

Beads incubated with 0, 10, or 100 μM of DHEA for 3 days were dissolved as previously described, and cell pellets were immediately lysed in lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM Na_3VO_4, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, and 1% Triton X-100 (all from Sigma), and centrifuged at 16 000×*g* for 10 min. Total protein in the supernatants was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin (BSA) as a standard. The syntheses of MMP-1, -3, and TIMP-1 were assayed in 50 μg samples of protein extracts using mouse anti-human MMP-1 Ab-1 (CloneX2A; Neomarkers, Fremont, CA, USA), mouse anti-human MMP-3 Ab-2 (Clone SL-1 IID4; Neomarkers), and mouse anti-human TIMP-1 Ab-2 (102D1; Neomarkers) as primary antibodies, and sheep anti-mouse IgG conjugated with horseradish peroxidase (HRP; Amersham Biosciences, UK) as a secondary antibody, and luminol as a chemiluminescent HRP substrate.

STATISTICAL ANALYSIS

All experiments were performed in triplicate. Results were expressed as means±standard deviation of three experiments. Statistical comparisons were made using Kruskal–Wallis test to examine differences between individual data points. Statistical significance was set at $P<0.05$.

Results

EFFECTS OF DHEA ON CELL PROLIFERATION AND GAG SYNTHESIS

The effect of DHEA on cell proliferation was examined at DHEA concentrations of 0, 10, 50, 100 μM after 1, 3, and 7 days of culture (Fig. 1). Chondrocytes at every concentration proliferated actively and achieved more than five population doublings by day 7. No significant differences were observed between cells treated with different DHEA concentrations at different times ($P>0.05$). These results demonstrate that DHEA treatment did not affect chondrocyte proliferation at any of these concentrations.

The effect of DHEA on GAG synthesis was examined by DMB assay at DHEA concentrations of 0, 10, 50, 100 μM after 1, 3, and 7 days of culture [Fig. 2(A–D)]. Total amount of GAG synthesis increased with time at all concentrations, but no statistical significance was found [$P>0.05$; Fig. 2(A)]. While GAG in further-removed matrix (FRM) did not seem to change apparently, GAG in media, and especially in CM, tended to increase with time [Fig. 2(B–D)]. However, no

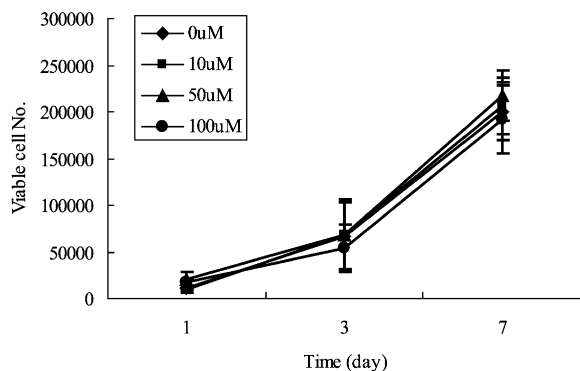

Fig. 1. The effect of DHEA on chondrocyte proliferation as determined by the MTS assay. Five thousand chondrocytes were initially plated in each well of a 96-well plate and treated with DHEA at 0, 10, 50, and 100 μM. On days 1, 3, and 7, the number of viable cells was measured by MTS assay. Experiments were performed in triplicate. Chondrocytes actively proliferated during culture, but no significant differences in viable cell numbers were observed at the different concentrations.

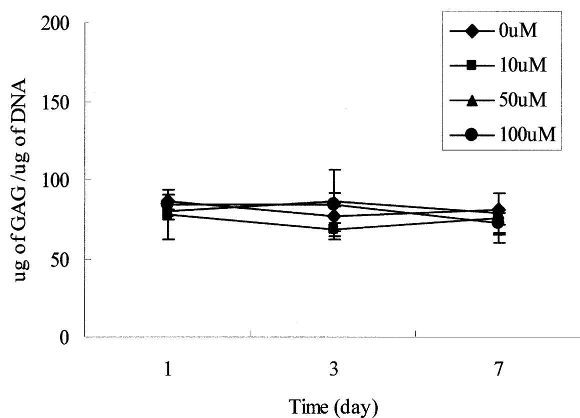

Fig. 2. The effect of DHEA on GAG synthesis as determined by the DMB assay. (A) Total GAG, (B) GAG in media, (C) GAG in FRM, (D) GAG in CM. Chondrocytes in alginate beads were treated with DHEA at 0, 10, 50, and 100 μM. On days 1, 3, and 7, the alginate beads were dissolved, and the total amount of GAG was determined by measuring the amount in media, supernatants, and pellets, and by summing them. (A) Total amount of GAG increased over the culture period of 7 days, but no significant difference was observed between the different concentrations or during the culture period. (B–D) In media, there was only small amount of GAG release from beads and it tended to increase over time. GAG amount in CM increased most prominently over the culture period in all beads at all concentrations, while that in FRM, it increased slightly.

statistical significances were found between different concentrations or between different time points during the culture period. These results suggest that DHEA, at least, does not impair the GAG production by chondrocytes.

EFFECTS OF DHEA ON THE GENE EXPRESSIONS OF TYPE I AND II COLLAGEN, MMP-1, -3, AND TIMP-1

Triplicate PCRs produced nearly identical results. The figures presented are representatives of the results obtained. PCR products of GAPDH were detected in all RNA preparations, which confirmed the uniformity of the RNA preparations. The mRNA expression of type I collagen was significantly suppressed by DHEA treatment at 50 and 100 μM ($P<0.05$), while that of type II collagen was significantly enhanced by DHEA treatment at the same concentrations ($P<0.05$) (Fig. 3). DHEA appeared to reduce the mRNA expression of type I in a dose-dependent manner. Type I collagen expression was maximally inhibited at a concentration of 100 μM and was reduced to 28% of that of the non-DHEA-treated control. Similarly, type II collagen expression was maximally enhanced at a DHEA concentration of 100 μM; up to 146% of that of the non-DHEA-treated control.

The effects of DHEA on the gene expressions of MMP-1, -3, and TIMP-1 were investigated with increasing concentrations of DHEA (Fig. 4). The gene expression of MMP-1 was significantly suppressed at concentrations of 50 and 100 μM ($P<0.05$), while the gene expressions of MMP-3 showed a similar suppressed pattern that was not of statistical significance ($P>0.05$). DHEA also significantly increased the gene expression of TIMP-1 at the lower concentration of 10 μM. The dose-dependent effect of DHEA was observed most prominently for the decreased gene expression of MMP-1, compared with those of MMP-3 and TIMP-1. The gene expression of MMP-1 was maximally suppressed to 48% of that of the non-DHEA-treated control at 100 μM, and that of MMP-3 was slightly suppressed to 82–93% of the control, regardless of DHEA concentrations. DHEA also enhanced the gene expression of TIMP-1 up to 120% of the control, although maximum gene expression occurred at 50 μM.

EFFECTS OF DHEA ON THE GENE EXPRESSIONS OF MMP-1 AND -3 IN THE PRESENCE OF IL-1β

IL-1β has been shown to induce the gene expression and the protein synthesis of metalloproteinases (MMPs), while it reduces only the synthesis of TIMP without affecting its gene expression[29]. After investigating the effects of DHEA on the mRNA expressions of MMP-1, -3, and TIMP-1, we studied whether DHEA treatment modulates IL-1β-induced gene expressions of MMP-1, and MMP-3. First, we determined the IL-1β concentration by culturing chondrocytes with increasing concentrations of IL-1β (0, 10, 100, and 1000 pg/ml) and measured the mRNA expressions of MMP-1 and -3 by RT-PCR as described previously. IL-1β was found to significantly induce the mRNA expressions of MMP-1 and -3 in a dose-dependent manner, and the maximal expressions of MMP-1 and -3 were 470 and 310% of that of the non-treated control, respectively, at 1000 pg/ml. Considering this result, we decided to use IL-1β at 1000 pg/ml.

Chondrocytes were cultured with 0, 10, 50, or 100 μM of DHEA and 1000 pg/ml of IL-1β for 3 days. DHEA significantly decreased IL-1β-induced gene expression of MMP-1 from the lowest concentration of 10 μM, dose dependently, to 74 and 53% of the non-treated control level at 10 and 50 μM, respectively (Fig. 5). The suppressive effect of DHEA plateaued above 50 μM of DHEA. DHEA at 50 μM also significantly decreased the IL-1β-induced gene expression of MMP-3, which was in contrast with the result obtained without IL-1β ($P<0.05$). The MMP-3 gene expression was maximally suppressed to 73% of that of the control at a DHEA concentration of 100 μM ($P<0.05$).

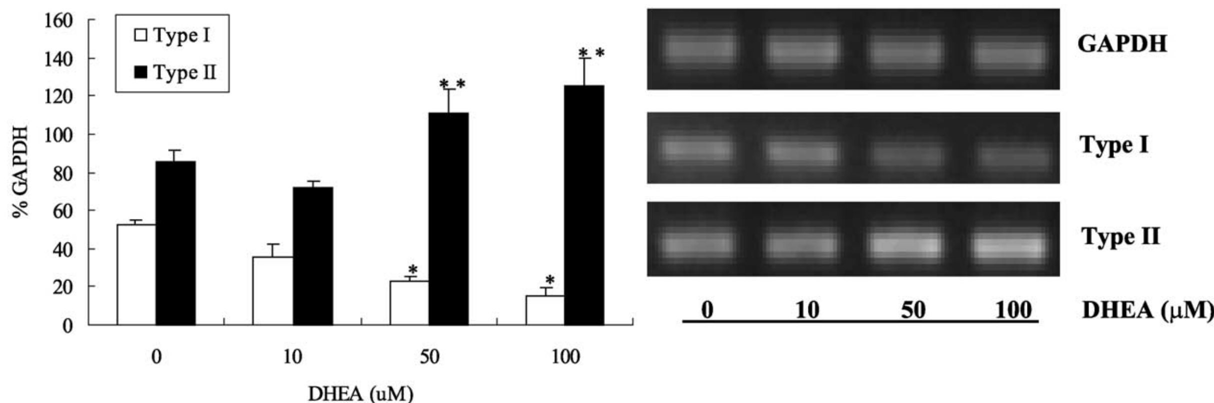

Fig. 3. The effects of DHEA on the gene expressions of type I and II collagen measured by RT-PCR. Chondrocytes in alginate beads were treated with DHEA at 0, 10, 50, and 100 μM for 3 days. Experiments were performed in triplicate and the bands shown represent typical results. Type I collagen gene expression was suppressed and type II collagen gene expression increased on increasing the DHEA concentration: *statistical significance in type I collagen expression ($P<0.05$), **statistical significance in type II collagen expression ($P<0.05$).

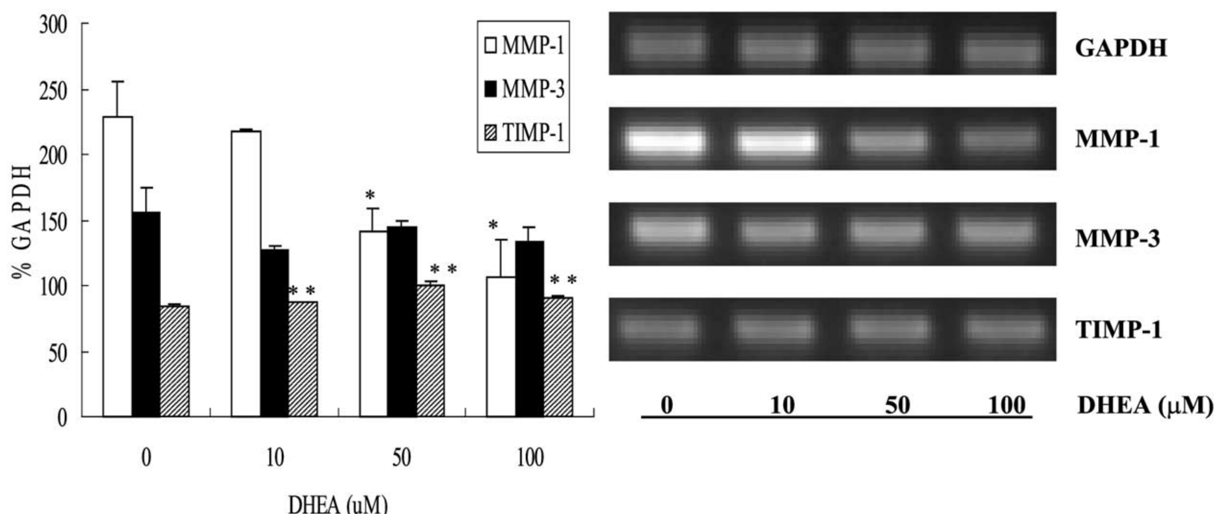

Fig. 4. The effects of DHEA on the gene expressions of MMP-1, -3, and TIMP-1 as determined by RT-PCR. Chondrocytes in alginate beads were treated with DHEA at 0, 10, 50, and 100 μM for 3 days. Experiments were performed in triplicate and the bands shown represent typical results. The gene expression of MMP-1 was significantly reduced at DHEA concentrations of 50 and 100 μM. The gene expression of MMP-3 was also reduced, but no statistical significance was found. The gene expression of TIMP-1 was elevated at DHEA concentration of 10 μM: *statistical significance in MMP-1 expression ($P<0.05$), **statistical significance in TIMP-1 expression ($P<0.05$).

EFFECTS OF DHEA ON THE PROTEIN SYNTHESIS OF MMP-1, -3, AND TIMP-1

To determine whether the suppression of MMP-1 and -3 mRNA levels and the enhancement of TIMP-1 mRNA levels are accompanied by an increase in protein synthesis, we assayed the production of mature MMP-1, -3, and TIMP-1 in the lysates of chondrocytes by Western blot analysis (Fig. 6). As was the mRNA expression, the protein level of MMP-1 was dose dependently reduced by treatment with DHEA, while that of MMP-3 was unchanged on increasing the concentration of DHEA. The translational level of mature TIMP-1 was significantly increased on increasing the concentration of DHEA, and this dose-dependent increase appeared more pronounced by Western blot analysis. Thus, it was found that the transcriptional effects of DHEA are associated with the translational activities for MMP-1, -3, and TIMP-1.

Discussion

This is the first study to demonstrate the *in vitro* effects of DHEA on osteoarthritic chondrocytes. We used alginate beads as a three-dimensional scaffold to culture chondrocytes, as they have been demonstrated to be compatible with chondrocytes[30,31]. In the present study, we analyzed the effects of DHEA on chondrocyte proliferation, GAG synthesis, the gene expressions, and protein syntheses of type I and II collagen, catabolic enzymes such as MMP-1, -3, and inhibitor of MMPs, such as TIMP-1, which are known to play important roles in the progression of OA. Furthermore, the effects of DHEA on IL-1β-induced gene expressions were also investigated. We found that DHEA has an ability to modulate the imbalance between MMPs and TIMP-1 during OA at the transcription level, which suggests that DHEA has a protective role against articular

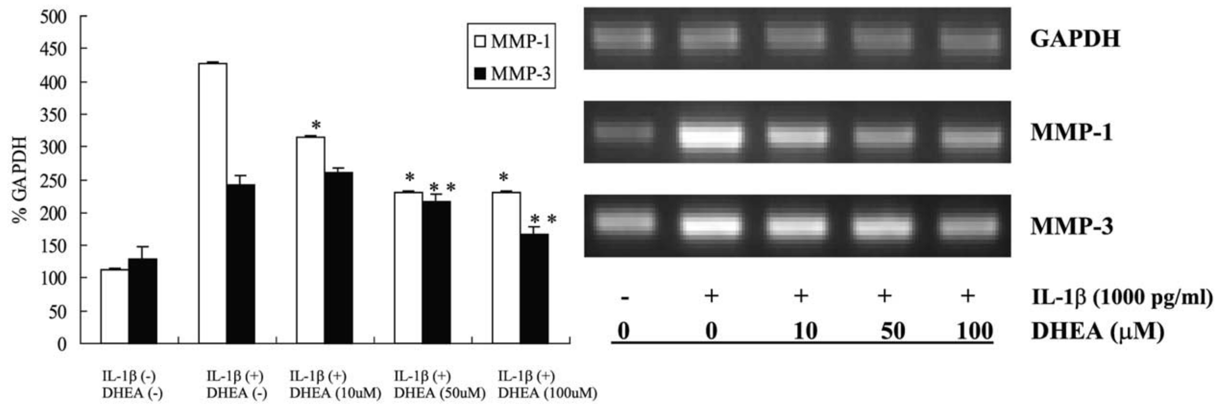

Fig. 5. The effects of DHEA on the gene expressions of MMP-1 and -3 in the presence of IL-1β measured by RT-PCR. Chondrocytes in alginate beads were treated with DHEA at 0, 10, 50 and 100 μM in the presence of 1000 pg/ml of IL-1β for 3 days. Experiments were performed in triplicate and the bands shown represent typical results. DHEA treatment suppressed the gene expressions of MMP-1 and -3 more so in the presence of IL-1β: *statistical significance in MMP-1 expression ($P<0.05$), **statistical significance in MMP-3 expression ($P<0.05$).

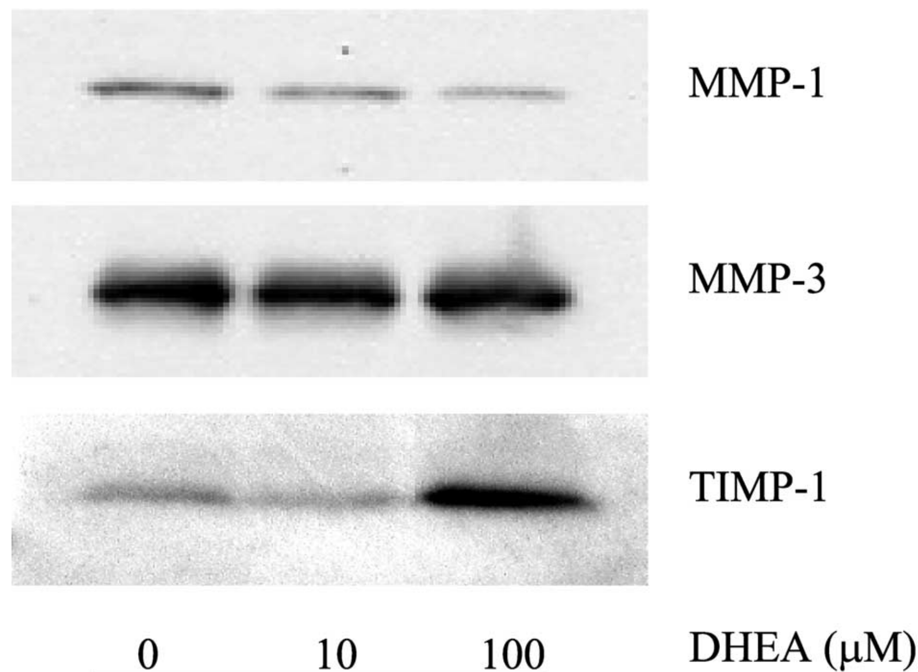

Fig. 6. The effects of DHEA on the protein syntheses of MMP-1, -3, and TIMP-1, as determined by Western blotting. Chondrocytes in alginate beads were treated with DHEA at 0, 10, and 100 μM for 3 days. Experiments were performed in triplicate and the bands shown represent typical results. The protein syntheses of MMP-1, -3, and TIMP-1 correlated with their gene expressions, indicating that DHEA acts at the transcription level.

cartilage loss. The present study demonstrates that DHEA treatment does not affect chondrocyte proliferation or GAG synthesis up to 100 μM. However, the gene expressions and syntheses of catabolic enzymes, such as MMP-1 and -3, were significantly suppressed by DHEA, while those of inhibitor of MMPs, such as TIMP-1, were significantly stimulated. Furthermore, the effects of DHEA treatment were accentuated by IL-1β treatment, as IL-1β-induced gene expressions of MMP-1 and -3 were reduced at lower concentration of DHEA.

In this work, DHEA treatment was not found to affect the proliferation or viability of chondrocytes, regardless of the DHEA concentration. We made use of the MTS method to measure the activities of mitochondrial dehydrogenases of viable cells. This method is known to produce results that are similar to those obtained by the [^{3}H]Thymidine uptake assay[32]. In addition, by using this method, we were able to measure the cytotoxicity of DHEA by directly measuring the number of viable cells.

A few reports have been concerned with the proliferative inhibitory effect of DHEA in other cell types and have suggested that the inhibition might occur via a DHEA-specific receptor that involves extracellular signal-regulated kinase 1 (ERK1) signaling pathways[33], via increasing G0–G1 cell populations and simultaneously decreasing the number of cells advancing to the S and G2–M cell cycle

phases[34], or via inhibition of glucose-6-phosphate dehydrogenase (G6PD)[35]. However, Yoneyama *et al.* reported that the growth response to DHEA *in vitro* was markedly affected by the culture conditions after experiments with vascular smooth muscle cells from human or rat aorta[36]. These divergent results reflect the current understanding of the effects of DHEA on cell proliferation, which demonstrably remains unclear.

The effects of DHEA on chondrocyte proliferation have not been previously reported and are described for the first time in the present study. On day 7 after culture for cell proliferation assay using the MTS assay, the number of the viable chondrocytes reached nearly 40 times that of day 1, which means that the cells proliferated more than five-fold. Also, cell proliferation was not affected by various concentrations used in the present study. This result indicates that chondrocytes can proliferate actively in the presence of DHEA, even at pharmacologic concentrations[26] (>10 μM) and that DHEA is not cytotoxic to chondrocytes.

In this *in vitro* study, DHEA was found to suppress the gene expression of type I collagen and to simultaneously increase the gene expression of type II collagen, both in a dose-dependent manner. Normally, type I collagen is not expressed in normal or osteoarthritic cartilage[37]. However, chondrocytes cultured as monolayer undergo a gradual dedifferentiation, characterized by a change from a spherical shape to a fibroblastic appearance. This occurs with changes in the types of collagen synthesized from type II into type I[38,39]. However, these changes have been reported to be reversed when dedifferentiated chondrocytes are transferred into alginate, i.e., type II collagen gene expression is upregulated and type I collagen gene expression is downregulated[40,41]. In the present study, isolated chondrocytes were cultured in monolayer for 7 days, which resulted in some dedifferentiation of chondrocytes. After transferring the monolayer-cultured chondrocytes to alginate beads, they began to gradually re-express the differentiated phenotype. DHEA accentuated the decreased gene expression of type I collagen and increased the gene expression of type II collagen, which means DHEA enhanced the re-expression of the chondrocyte phenotype in the alginate beads.

Various MMPs, including MMP-1 and -3 are known to be expressed in human[42] or experimentally induced OA[43–45], though their expression patterns are contradictory[46]. The activities of MMPs are regulated not only by gene expression, but also by the extracellular activation of proMMPs and by the inhibition of endogenous TIMPs inhibitors. In addition, cartilage destruction is believed to be caused by an imbalance between activated MMPs and TIMPs[47]. The expression of TIMPs has been thought to be relatively constant, regardless of the progression of experimentally induced OA[48], treatment with hyaluronic acid[49] or with non-steroidal anti-inflammatory drugs (NSAIDs)[50] or IL-1β[29]. However, dexamethasone and IL-1β treatments were found to reduce the expression of TIMPs[51]. We demonstrated that treatment with DHEA significantly suppresses the gene expression and protein synthesis of MMP-1, which suggests that DHEA influences at the gene level in the study. By contrast, DHEA increased the gene expression and protein synthesis of TIMP-1, indicating that DHEA has an anti-catabolic action, not only via MMPs suppression, but also via TIMP-1 induction.

The effects of DHEA were more prominent in the presence of IL-1β. DHEA suppressed the IL-1β-induced expressions of MMP-1 and -3 at 10 and 50 μM, respectively, which are lower concentrations when compared with the results in the absence of IL-1β. As IL-1β is well known to be elevated in osteoarthritic joints, these results indicate that DHEA could significantly reduce cartilage destruction in those joints via the suppression of the gene expressions of MMP-1 and -3, and by increasing the gene expression of TIMP-1, though we did not investigate the effect of DHEA on the IL-1β-induced expression of TIMP-1. We did not investigate the effect of DHEA on protein synthesis of MMP-1, -3, and TIMP-1 in the presence of IL-1β. However, because a study on the effect of DHEA on protein synthesis should include investigations on the activity of those enzymes, and not only the protein levels, it seems to be beyond the scope of this study, suggesting future studies.

The mechanisms of DHEA action in chondrocytes are not clear. However, DHEA has been known to have anti-inflammatory effects, both in cells other than chondrocytes and/or *in vivo* via the inhibition of proinflammatory cytokine secretion, such as TNF[52,53], IL-1[54], and IL-6[55]. Moreover, the serum level of DHEA was found to be negatively correlated with serum IL-6 in human[56]. The common mechanism of the anti-inflammatory action of DHEA seems to concern the direct inhibition of nuclear factor kappa B (NF-κB) transfer to the nucleus[57] via the activation of peroxisome proliferator activated receptor alpha (PPARα), the inhibition of the binding of the nuclear factor activator protein 1 (AP-1) to the DNA[58], and peripheral conversion to androgens and estrogens[59]. From this point of view, it is interesting to note that the modulation of the gene expressions of MMPs by IL-1 in chondrocytes is likely to be mediated by serine–threonine kinases of the mitogen-activated protein kinase (MAPK) family[60], which comprises ERK, the c-Jun N-terminal kinase (JNK), and p38. As the endpoint of MAPK activation is the production of phosphorylated active AP-1 transcription factor, which is composed of c-Jun and c-Fos, the effect of DHEA on the suppression of MMPs, presumably, and at least in part, is likely to be exerted via the inhibition of the MAPK kinase pathway. In addition, as IL-1 increases the activity of NF-κB, a transcription factor that regulates numerous proinflammatory genes, such as MMP-1 and -3[61,62], the inhibitory effects of DHEA may blockade the IL-1-induced activity of NF-κB. Further studies are needed to elucidate the precise role of DHEA in the production of chondrocyte MMPs.

This is the first study on the effects of DHEA on human osteoarthritic chondrocytes. Our results demonstrate that DHEA does not exert significant influence on cell proliferation or viability, or GAG production. On the other hand, DHEA does suppress the gene expressions and protein syntheses of MMP-1 and -3 and increase those of TIMP-1. Furthermore, the inhibitory effects of DHEA on the gene expressions of MMP-1 and -3 were found to be accentuated in the presence of IL-1β.

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