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Original Article

The Efficiency of Deer Antler Herbal Acupuncture on Modulation and Prevention of IL-1 Mediated Activation in Rat Chondrocytes at a Receptor Level

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Abstract

Objectives : Deer antler Herbal-Acupuncture (DHA) solution represents one of the most commonly used medicine to treat rheumatoid arthritis. But, mechanisms of its antiarthritic activities are still poorly understood. Identification of common DHA aqua-acupuncture capable of affording protection or modulating the onset and severity of arthritis may have important human health implications.

Results : We determined if DHA could prevent the binding of IL-1β to its cellular receptors. DHA addition to rat chondrocytes treated with IL-1β or with reactive oxygen species(ROS) prevents the activation of proteoglycan synthesis. After treatment with IL-1β, DHA increased the expression of mRNA encoding the type II IL-1β receptor. These results emphasize the potential role of two regulating proteins of the IL-1β signaling pathway that could account for the beneficial effect of DHA in osteRArthritis. The present study also identifies a novel mechanism of DHA-mediated anti-inflammatory activity.

Conclusion : It is shown that DHA inhibits both IL-1β- and TNF-α-induced NO production in normal human articular chondrocytes. The observed suppression of IL-1-induced NO production is associated with inhibition of inducible NO synthase(iNOS) mRNA and protein expression. In addition, DHA also suppresses the production of IL-1-induced cyclooxygenase-2 and IL-6. The constitutively expressed cyclooxygenase-1, however, was not affected by the sugar. These results demonstrate that DHA expresses a unique range of activities and identifies a novel mechanism for the inhibition of inflammatory processes.

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Ⅰ. Introduction

Reumatoidarthritis(RA) is characterized by cartilage alterations, such as quantitative and qualitative modifications of proteoglycans(PGs) and collagens. An imbalance between the biosynthesis and the degradation of matrix components leads to a progressive destruction of the tissue, resulting in extensive articular damage[7]. Cartilage damage in RA is known to be largely mediated by interleukin 1 (IL-1), a cytokine that initiates a number of events leading to cartilage destruction, including the inhibition of matrix macromolecule biosynthesis and the increase of catabolic pathways.

Many studies have demonstrated that cartilage from patients with RA is characterized by accelerated turnover of the cartilage matrix components and by inadequate repair[8-9].

Since IL-1β treatment is well known to lead to the production of reactive oxygen species(ROS) by cells, we hypothesized that DHA could prevent the action of IL-1β by antioxidant effects. Secondly, we wanted to know if the DHA could prevent the binding of IL-1 to its cell surface receptors, or could interfere at post-receptor levels.

Therefore, the objectives of this study were (i) to look for a potential antioxidant effect of DHA by incubating chondrocytes with a ROS-generating system and comparing the results with those obtained in IL-1-stimulated cells and (ii) to determine if DHA could modulate the binding of IL-1 on its receptor via a possible increase in type II IL-1 receptor (IL-1RII) which is a decoy receptor, or an increase in IL-1 receptor antagonist (IL-1ra). The current study presents experimental evidence that DHA possess a unique range of anti-inflammatory activities and inhibit NO, and IL-6 production induced in cultured human articular chondrocytes by IL-1.

Ⅱ. Materials and methods

1. Materials

It was studied that the effects of DHA prepared from the pilose antler of Cervus korean TEMMINCK var. *mantchuricus Swinhoe*(DHA), a traditional immunosuppressive and immuno-activating Korean aqua-acupuncture, on collagen-induced rat rheumatoid arthritis(RA) model dried DHA was solutioned twice with hot water and the combine solution was concentrated and the total soluble fraction was concentrated under vacuum, dissolved in water, and freeze-dried. This light brown, solid matter is called DHA. A solution of 0.2% DHA in the water was prepared and given to experimental mice ad libitum as the sole source of aqua-acupucture

DHA tablets, a water solution of DHA were purchased from Kyungju Oriental Medical Hospital, Dongguk University(Kyungju, Korea) as an i.p injection grade for human. Each tablet contained 100㎍ of the solution. For i.p. injection into rats, randomly selected tablets were ground and suspended in normal saline at a concentration of 50㎍/10㎕.

The animals receiving normal phosphate buffered saline (PBS) served as the non-DHA- injected group. This DHA feeding protocol has been used in rat in many prior studies from this and other laboratories[23].

2. Methods

1) Chemicals

Dulbecco's modified Eagle's medium (DMEM),

fetal calf serum (FCS), L-glutamine, gentamicin, reverse transcriptase, and restriction enzymes were obtained from Gibco-BRL (Seoul, Korea). Taq polymerase was supplied by Takara (Seoul, Korea), and the nucleotides were synthesized by Core-Bio (Seuol). Recombinant human IL-1β was purchased from Biosystem (Korea).

2) Rat Chondrocyte Cultures and Treatments

Male Wistar rats (130-150g) (KCTC) were housed under controlled temperature and lighting conditions with food and water ad libitum. Articular cartilage, isolated from femoral head caps, was aseptically dissected, and chondrocytes were obtained after digestion of the cartilage fragments in pronase (2% w/v in 0.15 M NaCl) followed by an overnight digestion in collagenase B (1.5% w/v in DMEM without serum)[11]. The experiments were performed with first-passage cultures, 6 days after collagenase treatment. For this purpose, chondrocytes were grown to confluence for 6 days in 25㎠ flasks (about 4×106 chondrocytes per flask) in 5㎖ of complete medium (DMEM supplemented with 2 mM L-glutamine, 50 g/㎖ gentamicin, 10% FCS v/v). Cells were then cultured for 6 h in FCS-free medium containing glucose (4.5g/ℓ) or DHA(10 ㎍/㎖), and finally stimulated with IL-1β (25 or 250 U/㎖) for 16 hrs.

Cartilage shavings were harvested by the tissue banks within 24 post mortem, placed in tissue culture medium (DMEM, 10% FBS, penicillin, streptomycin), and shipped to the laboratory at 4°C. This tissue was processed in the laboratory within 24 hrs after harvest.

Chondrocytes were isolated from the cartilage by collagenase digestion and maintained in continuous monolayer cultures in DMEM containing 10% FBS. Cell viability after chondrocyte isolation by collagenase digestion of normal cartilage is > 95%. This level is maintained for at least 96 h post mortem. Studies on IL-1 effects as a catabolic response showed no apparent changes as a function of variations in the time between death and tissue processing when NO and IL-6 release were measured. Experiments reported in this work were performed with primary or first passage cells.

For stimulation with a ROS-generating system, cells were incubated for 30 min in a DHA-containing medium in the presence of hypoxanthine (4 mM) and xanthine oxidase (10 mU/㎖)[11]. Cells were then cultured in glucose- or DHA-containing medium for 8 hrs (mRNA expression determination) or for 16 hrs (PG synthesis measurement). For the determination of proteoglycan synthesis, chondrocytes were entrapped into alginate beads as follows. The cells were suspended in sterile filtered low viscosity alginate solution (1.2% w/v) at 6×106 cells/㎖ and slowly expressed through a 22 gauge needle into a 100 mM CaCl2 solution. After two washes with 0.15 M NaCl, beads were cultured for 6 days in complete medium containing 4.5 g/ℓ of glucose. Before stimulation with ROS or IL-1, cells were cultured with complete medium with 2% FCS, for 8 h in the presence of 4.5 g/ℓ of glucose or DHA.

3) Measurement of PG Biosynthesis

The incorporation of radiolabeled sodium sulfate into PGs in rat chondrocytes encapsulated into alginate beads was measured 16 h after ROS or IL-1β stimulation. Alginate beads were incubated in complete medium (4.5g/ℓ glucose or glucosamine) containing 2% FCS and supplemented with 10 uCi/㎖ of Na_2 $35SO_4$ for 4 hrs at 37°C. The encapsulated chondrocytes were washed several times with 0.15 M NaCl and solubilized in Soluene 350(0.5 M quaternary ammonium hydroxide in toluene) overnight. The amount of radiolabeled sulfate incorporated, considered a reliable evaluation of the amount of newly synthesized sulfated glycosaminoglycans, was quantified by liquid scintillation.

4) Analysis of IL-1RII mRNA Expression by quantitative Reverse Transcriptase Polymerase Chain Reaction(RT-PCR)

Total RNA was isolated from cell cultures by a single-step guanidinium thiocyanate-phenol chloroform method using Trizol (Life Technologies) according to the manufacturer's protocol. mRNA expression by rat chondrocytes was analyzed using a quantitative multistandard RT-PCR, a method that takes advantage of sequence conservation of both the gene of interest and β-actin between animals species[12]. The protocol allowed us to normalize the amount of mRNA of the gene of interest with respect to that of β-actin mRNA in each sample. Total RNA samples solutioned from rat cells were mixed with a constant amount of RNA prepared from mouse tissues, which brought the sequences of both competitive mouse β-actin and the gene of interest and thus acted as a multistandard source.

For amplification of IL-1RII, the direct primer extended from nucleotides 401 to 421, and the reverse primer extended from nucleotides 723 to 744 in accordance with the rat sequence (GenBank accession number Z22812). EcoRI digested the mouse IL-1RII product into 2 fragments (228 and 115 bp), while the rat product remained uncut[13].

Digested RT-PCR products were resolved by agarose gel electrophoresis stained with ethidium bromide. The bands were visualized under UV light and photographed by a computer-assisted camera. Quantitation of each band was performed by densitometry analysis with NIH software. Results are expressed as the ratio (analyzed generat/-actinrat)×(-actinmouse/analyzed genemouse), in arbitrary units.

5) Quantification of Nitrites

Chondrocytes were plated at 40,000 cells/well in 96-well plates in the presence of 1% FBS. After 48 h, the medium was changed, and the cells were stimulated with IL-1 (Sigma) at a concentration of 5 ng/㎖ for 24 h. NO production was detected as NO_2 accumulation in the culture supernatants by the Griess reaction.

6) IL-6 Measurement

IL-6 in the culture supernatants[14] was measured by ELISA (R&D Systems, Minneapolis, MN) in accordance with the supplier□□s protocol.

7) Statistical Analysis

After comparison of data by analysis of variance, the different groups were compared using Fisher's t-test. Assays were made in triplicate. P values less than 0.05 were considered significantly.($p<0.05$)

Statistical analysis of the generated data was also performed with the aid of StatMost 32 program for Windows (Dataxiom Software, Los Angeles, CA).

Ⅲ. Results

1. DHA effect of ROS or IL-1β on PG Synthesis in Rat Chondrocytes Cultured in the Presence of Glucose or DHA

Since IL-1β and tumor necrosis factor have been reported to increase ROS in chondrocytes[15], thus decreasing the biosynthesis of PG, we hypothesized that glucosamine could reverse both the ROS and the IL-1β effects. For this reason, the concentration of the hypoxanthine/xanthine oxidase system (4 mM, 10 mU/㎖) was chosen to produce approximately similar reductions of PG synthesis to that produced by 250 U/㎖ IL-1 treatment.

Treatment of chondrocytes with ROS or IL-1β in glucose medium resulted in a significant decrease in PG synthesis (-23% for ROS and

-25% for IL-1, P<0.05; Fig. 1). However, in contrast to what we observed with IL-1β treatment, addition of DHA provided less significant prevention against the ROS-mediated inhibitory effects on PG synthesis (-28.5%, P<0.05; Fig. 1). Moreover, DHA induced a PG synthesis decrease by itself (-39%, P<0.05; Fig. 1). DHA was only able to counteract the further inhibition of PG synthesis caused by IL-1β treatment but not after ROS challenge. The decrease in PG synthesis induced by ROS production could be related to different cellular pathways than those involved in the IL-1β challenge.

Cells were cultured in alginate beads in 2% FCS medium containing glucose (4.5 g/ℓ) or DHA (10 ug/mℓ). ROS were produced as reported: in brief, cells were cultured for 6 h in serum-free medium containing glucose or DHA before being incubated for 30 min in the same medium in the presence of hypoxanthine (4 mM) and xanthine oxidase (10 mU/mℓ) and 16 h later, radiolabeled sulfate incorporation was analyzed as described in text.

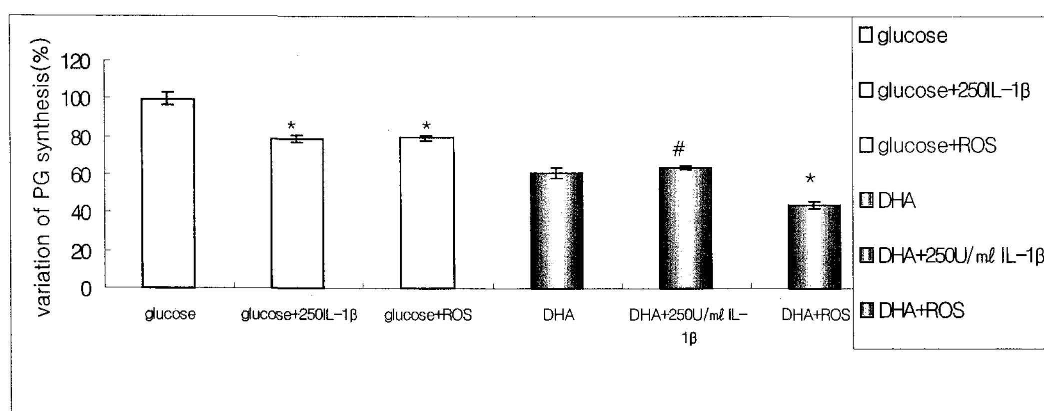

Fig. 1. DHA effect of ROS or IL-1β on PG Synthesis in Rat Chondrocytes Cultured in the Presence of Glucose (white bars) or DHA (gray bars)

Results are expressed as percentage of variations by comparison to the control (value of 100 for glucose medium) and are the mean±S.D. of three different assays (n=3; *p<0.05 versus respective control, #p<0.05 versus 4.5 mg/mℓ glucose medium).

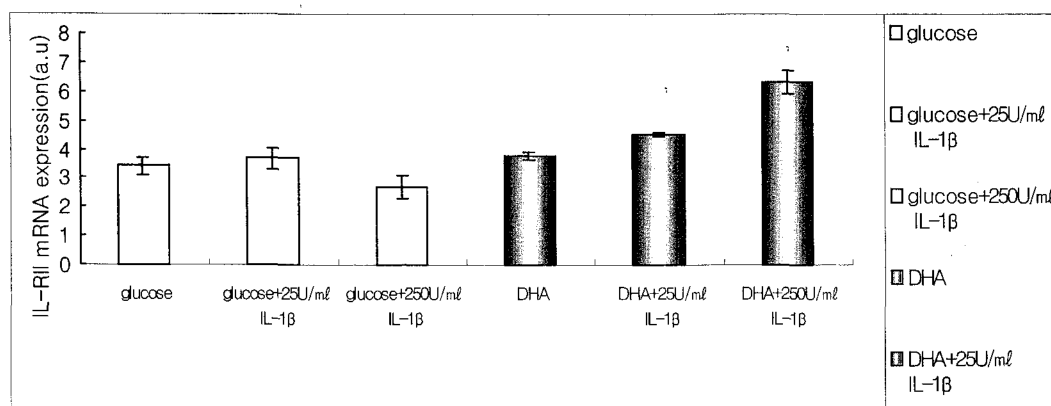

Fig. 2. Effect of IL-1β Treatment on IL-1RII mRNA Expression in Rat Chondrocytes in the Presence of Glucose (white bars) or DHA (gray bars)

Results are expressed in relative arbitrary units, and are the mean±S.D. of three different assays (n=3; *p<0.05 versus respective control).

2. Effect of IL-1β Treatment on IL-1RII mRNA Expression in Rat Chondrocytes in the Presence of Glucose or DHA

We therefore investigated the influence of DHA on the mRNA expression of the decoy receptor IL-1RII in chondrocytes treated or not with IL-1β. The addition of IL-1β (25 U/㎖ or 250 U/㎖) for 12 hrs to a culture medium containing glucose did not induce any variation of IL-1RII mRNA expression (Fig. 2). On the other hand, the IL-1β -mediated stimulation of cultured rat chondrocytes in the presence of DHA led to a dose-dependent increase in the expression of this mRNA, reaching 51% at the higher dose of the cytokine (Fig. 2). No significant difference was observed between controls containing glucose and glucosamine, in the absence of IL-1β. We also evaluated the effects of IL-1β on IL-1ra mRNA expression. In contrast to IL-1RII, no variation in IL-1ra mRNA expression was measured in the presence of glucosamine (data not shown). Cells were cultured for 6 hrs in FCS-free medium containing glucose (4.5 ug/㎖) or DHA (10 ug/㎖), and finally stimulated with IL-1β- (25 U/㎖ or 250 U/㎖) for 16 hrs.

3. Effect of DHA on IL-1-induced NO Production in Cultured Human Articular Chondrocytes

IL-1 is known as a potent inducer of NO production in cultured human articular chondrocytes[16]. We demonstrated that both DHA was capable of suppressing NO production triggered by IL-1 (Fig. 3). The differences between NO production in chondrocyte cultures stimulated with IL-1 and chondrocyte cultures stimulated with IL-1 plus DHA was statistically significant, $p < 0.001$ Maximal inhibitory effect of DHA was observed with a concentration of 20 ug/㎖; concentrations lower than 1 ug/㎖ were insufficient in the suppression of NO production (Fig. 4). The IC50 for DHA was 13.2 ± 3.2 mM, $p < 0.01$ DHA at doses up to 20 ug/㎖ did not affect cell viability measured by MTT assay[17]

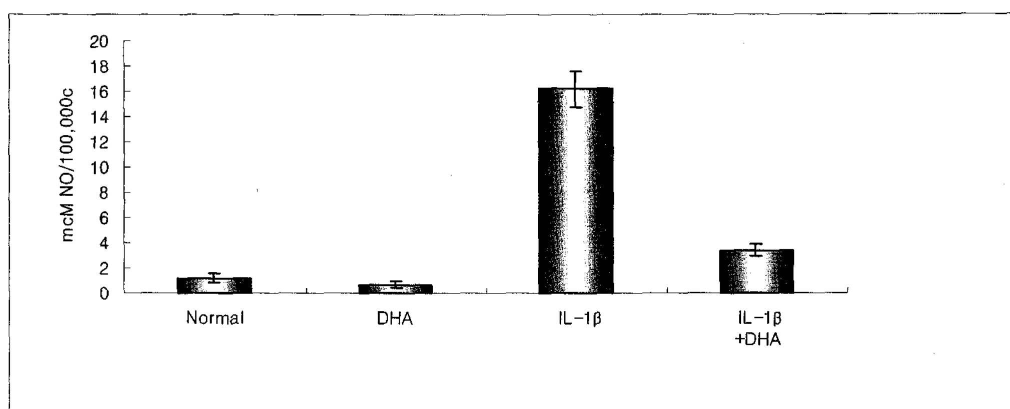

Fig. 3. Effect of DHA on IL-1-induced NO Production in Cultured Human Articular Chondrocytes
Human articular chondrocytes were stimulated with IL-1β (5 ng/㎖) and incubated with various concentrations of DHA 24 hrs. NO production was measured in culture supernatants by the Griess reaction. This figure represents mean ± SEM of the data obtained from seven independent experiments using seven different chondrocyte donors. CTRL, control.

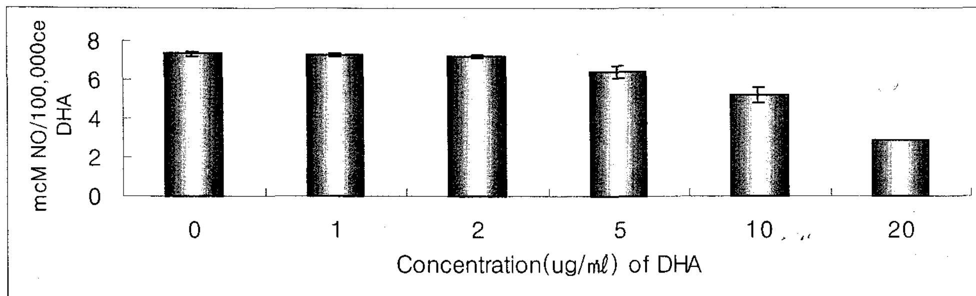

Fig. 4. Dose Response of IL-1β-mediated NO Production to DHA in Human Articular Chondrocytes
Human articular chondrocytes were stimulated with IL-1β(5 ng/mℓ) and incubated with DHA(10 ug/mℓ) for 24 h. NO production was measured in culture supernatants by the Griess reaction.

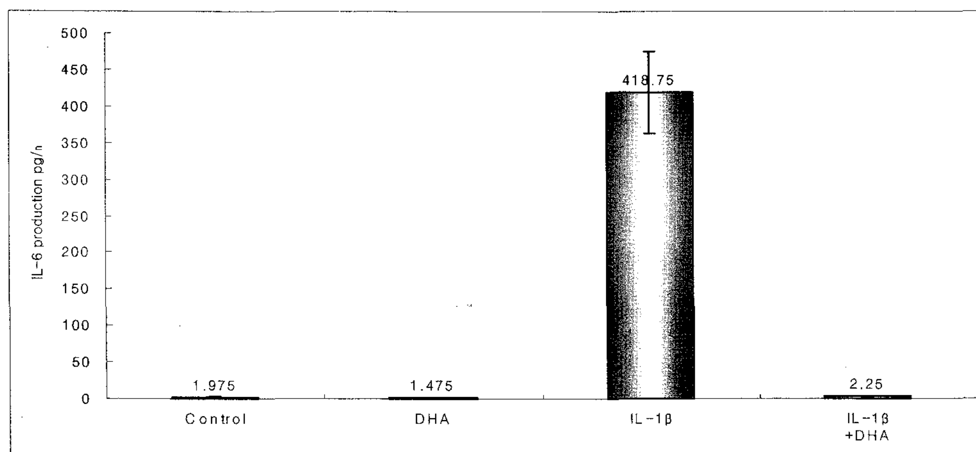

Fig. 5. Effect of DHA on IL-1-induced IL-6 production in cultured human articular chondrocytes
Human articular chondrocytes were stimulated with IL-1 (5 ng/mℓ) and incubated with DHA (10 ug/mℓ) for 24 hrs. Production of IL-6 was measured in culture supernatants by ELISA. This figure represents mean ± SEM of the data obtained from three independent experiments using three different chondrocyte donors. CTRL, Control.

4. Effect of DHA on IL-1-induced IL-6 Production by cultured Human Articular Chondrocytes

DHA was capable of inhibiting IL-6 production in cultured human articular chondrocytes stimulated with IL-1 (Fig. 5). The differences were statistically significant($p < 0.001$) Therefore, DHA is capable of suppressing several IL-1-inducible products of inflammation, but does not inhibit constitutively expressed molecules.

Ⅳ. Discussion

Reumatoidarthritis(RA) is the most common joint disorder and has an immense socioeconomic impact.[1-3] However, the conservative treatment of RA is still limited to a few classes of medications, such as acetaminophen, nonsteroidal anti-inflammatory drugs, injectable intraarticular corticosteroids, and hyaluronic acid, which provide primarily pain relief, but have not yet been demonstrated to interfere with the progression of the disease[4-6].

Unossified horn or pilose antler cut from deer which belong to the Cervidae is generally termed "Nokyong". Nokyong is one of the most famous Korean traditional medicines and has been considered to possess sexual-reinforcing and anti-aging actions. Thus, DHA has been used

invigorate the kidney-yang, replenish vital essence and blood and strengthen muscle and bones in a traditional Korean medicine. Solution of DHA, prepared from the pilose antler of *Cervus korean* TEMMINCK var. *mantchuricus Swinhoe* (DHA), a traditional immunosuppressive and immuno-activating Korean aqua-acupuncture, have sometimes been compounded in recent Korean commercial restoratives, although little is yet known about the pharmacological effects or active ingredients. solution from DHA by water boiling methods, has been widely used in the treatment of some immune-related diseases, especially rheumatoid arthritis(RA) and satisfactory results are obtained[21-22]. However, little is known about the mode of action of this traditional medication on RA.

DHA represents a new generation of medicine, which possess potentially chondroprotective or disease-modifying properties, and were originally suggested to promote the repair of damaged cartilage. Since DHA can be used as a single pharmacologic agent to treat RA[10] the preparation has gained considerable popularity, and now is being consumed by many RA patients. Despite the increased use of DHA in the treatment of RA, the mechanisms accounting for its in vivo and in vitro activity are still far from clear.

Some symptomatic slow-acting drugs, such as DHA, have been shown to be effective in relieving the symptoms of RA. Reports of symptomatic relief afforded by DHA on the treatment of RA have spurred new research into its mechanism of action on cartilage[10].

DHA solution is widely used in the long time management and the treatment of RA, particularly, in Korea. But, the mechanism by which the DHA solution modify the clinical status of RA are not well understood.. To investigate the effects of DHA on ROS-mediated or IL-1β-mediated PG synthesis decrease, rat chondrocytes were isolated, maintained in culture in the presence of glucose or DHA and challenged with ROS or IL-1β.

Previously, DHA solution inhibited production of IL-1β and TNF-α from macrophags in response to in vivo stimulation with bacterial lipopolysaccharides when the solution was administered into rat once a day for 7 days[12], suggesting that the DHA ectract administered orally into the patients inhibit cytokine production from both T cells and macrophages and potent effects on RA. Therefore, in this study, we examined the influence of DHA solution on cellular immune responses by using rat collagen induced arthritis(CIA), an experimental model for RA. The present results clearly demonstrated that the solution strongly inhibits T-cell activation including blastogenesis and cytokine production in response to antigenic stimulation in vitro. Furthermore, macrophage activation, was also suppressed by the DHA solution. It was observed that the DHA injection has significant reductive effects on the development of CIA in rats at dosages of 100-150μg/kg/week. DHA treatment also suppressed the production of the proteases of cytoplasmic, lysosomal and matrix protease types.

DHA solution might be a useful tool for the treatment of RA. It would be incredible if the drugs as powerful as this did not have seriuos toxicity, but further studies will be necessary to answer this question. However, biochemical and metabolic analysis of the constituents of DHA ectract have to be analysed in further delineating its mechanisms of action in arthritis.

Despite the fact that DHA represents one of the most commonly used drugs to treat RA, the molecular mechanisms of its activity are still poorly understood. Available experimental data indicate that DHA possesses both chondroprotective and anti-inflammatory effects. The chondroprotective action of DHA manifests as accelerated of glycosaminoglycan synthesis in cultured chondrocytes and cartilage tissue.

Anti-inflammatory mechanisms, besides DHA-induced up-regulation of glycosaminoglycan synthesis, are probably contributing to its antiarthritic activities as well. DHA had anti-inflammatory activity and protected rats from paw edema induced by bradykinin, serotonin, and histamine.

DHA did not suppress COX or proteolytic enzymes in the inflamed rat paw, but it did suppress superoxide generation and lysosomal enzyme activities in rat liver[10].

RA affects approximately 12% of the population, and the incidence increases with age. Current treatments for RA are limited to short-term symptom control with acetaminophen and non-steroidal anti-inflammatory drugs, which do not slow or reverse the degenerative process. DHA has recently received a great deal of attention from the public as a potential treatment for RA[10] since this drug has been proposed to slow down and possibly reverse these degenerative processes. However, the mechanisms of the beneficial effect of DHA on cartilage disease are still unknown. We showed that DHA not only corrected these effects but also antagonized the production of various pro-inflammatory mediators activated by IL-1β, such as NO and prostaglandin E2. Altogether, these results suggested that the pharmacological effects of DHA in the treatment of RA would involve a modulating action of this DHA at some stages of IL-1β signaling events.

There are many potential ways in which DHA could, directly or indirectly, modify IL-1β signaling pathways. The protective effects of DHA have been considered at three separate levels: (i) towards the effects induced by radicals and (ii) the variations of mRNA encoding the expression of the decoy receptor IL-1RII and the receptor antagonist IL-1ra, and known to be involved in inflammation. IL-1β induces a cellular stress that can generate ROS production, which, in turn, could activate the NF-B pathway by interacting with NIK[19]. We hypothesized that the presence of ROS could explain the decrease in PG synthesis. If DHA reversed the IL-1β-mediated decrease in PG synthesis, no effect was observed on ROS-mediated variations in biosynthesis of PG. These data suggest that ROS and IL-1β decreased the PG synthesis via two different pathways.

Two types of IL-1β receptors have been described. The inflammatory effect of IL-1β requires signaling through the cytoplasmic domain of IL-1RI. The decoy IL-1RII can also interact with IL-1β. However, as a truncated protein, its interaction with the cytokine is unable to generate signaling. By trapping part of IL-1β, the decoy receptor can modulate its concentration and effects. This property makes IL-1RII a potential target in order to block the IL-1β signaling pathways[17]. Many studies suggest that IL-1RII plays an important physiological role in the regulation of IL-1β action in the inflammation sites[18]. Several drugs, such as aspirin or glucocorticosteroids, could induce this decoy receptor, thus decreasing inflammation by preventing the binding of IL-1β to target cells[19-20]. Our study showed that the mRNA expression of IL-1RII was not changed in rat chondrocytes challenged with IL-1β in the presence of glucose. However, the expression of the decoy receptor was significantly increased in chondrocytes cultured in the presence of DHA, but only when the cells were treated with IL-1β. By increasing IL-1RII mRNA expression, DHA is able to reduce the binding of IL-1β on IL-1RI and therefore modulates its effects. However, at this state of the work, we are unable to explain by which mechanism DHA induces the increase in IL-1RII expression in IL-1β-treated chondrocytes.

The present study is the first to examine the effect of DHA on human chondrocyte response toward the stimulation with IL-1, and it describes a novel mechanism of DHA-mediated anti-inflammatory activity. Results of our experiments clearly indicated that DHA is capable of inhibiting IL-1-induced NO production in cultured human articular chondrocytes. In addition to its NO-inhibitory activity, DHA also suppressed the production of IL-1-induced IL-6.

DHA did not suppress all responses in chondrocytes induced by IL-1. Moreover, it was synergistic with IL-1 in the induction of TGF1 (data not shown). Collectively, these findings suggest that DHA selectively inhibits cytokine-

induced gene expression and the production of certain proinflammatory mediators.

Several aspects of the discovered DHA-mediated activity require more detailed discussion. Our experiments showed that both DHA in the lower range measurably inhibited NO production; concentrations below 1 ㎍/㎖ were not effective.

DHA has several potential advantages as a potential therapeutic anti-inflammatory agent. The present study also addressed potential mechanism involved in DHA-mediated inhibition of the IL-1 response. One possibility is a DHA-mediated inhibition of phosphorylation events in the IL-1 signaling cascade.

In conclusion, the study demonstrates that DHA expresses anti-inflammatory and chondroprotective activities by interfering with cytokine-inducible gene expression in chondrocytes.

The present work also demonstrates that DHA acts, at least, at different levels in rat chondrocytes of IL-1RII expression. Thus, these results contribute to clarify the mechanisms of action of DHA on cartilage.

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