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## Dihydrotestosterone Inhibits Interleukin-1 $\alpha$ or Tumor Necrosis Factor $\alpha$ -Induced Proinflammatory Cytokine Production *via* Androgen Receptor-Dependent Inhibition of Nuclear Factor- $\kappa$ B Activation in Rheumatoid Fibroblast-Like Synovial Cell Line

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**Rheumatoid arthritis (RA) is a disease with significant gender differences in its prevalence and clinical features. Interleukin (IL)-1 and tumor necrosis factor (TNF)  $\alpha$  produced by synoviocytes are principle inflammatory and destructive mediators of RA. We found that a potent androgen, 5 $\alpha$ -dihydrotestosterone (DHT) inhibits IL-1 $\alpha$ -induced production and mRNA expression of IL-8, IL-6 and IL-1 $\beta$  from RA patient-derived fibroblast-like synovial cell line MH7A. Promoter analysis of the *IL-8* gene revealed that nuclear factor (NF)- $\kappa$ B activation is critical for its transcriptional activation by IL-1 $\alpha$ , and DHT inhibited the IL-1 $\alpha$ -induced NF- $\kappa$ B activation in a manner dependent on the androgen receptor (AR). DHT also inhibited the effects of TNF $\alpha$  on the cells over-expressed with AR, indicating that sufficient expression level of functional AR was necessary for the inhibitory effect of DHT on TNF $\alpha$ . These results suggest that androgen contributes to the prevention against RA and its gender difference by inhibiting IL-1 $\alpha$  or TNF $\alpha$ -induced proinflammatory cytokine production from synovial fibroblast-like cells by inhibiting NF- $\kappa$ B activation in a manner depending on AR.**

**Key words** rheumatoid arthritis; synoviocyte; androgen; interleukin-1 $\alpha$ ; tumor necrosis factor  $\alpha$ ; interleukin-8

Rheumatoid arthritis (RA) is a disease resulting from a combination of environmental and genetic factors,<sup>1</sup> and is characterized by the chronic inflammation and hyperproliferation of synovial cells in multiple joints. Proinflammatory cytokines and chemokines produced by synoviocytes and infiltrating immune cells are implicated in the disease pathogenesis.<sup>2</sup> In synovial fluids of RA patients many cytokines and chemokines are present, including interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-3, IL-4, IL-6, IL-8, IL-15, IL-17, interferon (IFN)  $\gamma$ , tumor necrosis factor (TNF)  $\alpha$ , TNF $\beta$ , transforming growth factor  $\beta$ , granulocyte-macrophage colony-stimulating factor and macrophage inflammatory protein-3 $\alpha$  (MIP-3 $\alpha$ ) (CCL20).<sup>3,4</sup> Among these cytokines, TNF $\alpha$ , IL-1 and IL-6 are pivotal in the pathogenesis of the disease. IL-1 and TNF $\alpha$  increase the induction of cytokines, chemokines, proteases, prostaglandin E<sub>2</sub>, reactive oxygen intermediates, proliferation of synovial fibroblasts, cartilage degradation, infiltration of inflammatory cells, angiogenesis and fever.<sup>5,6</sup> IL-1 is a major cytokine inducing cartilage degradation, and mediates cartilage degradation by TNF $\alpha$ .<sup>7</sup> IL-6 induces T cell growth, stimulates B cells and is a potent inducer of acute phase proteins.<sup>8</sup> Indeed an antibody against TNF $\alpha$ , a soluble receptor for TNF $\alpha$ , interleukin-1 receptor antagonist (IL-1ra) and an antibody against IL-6 appeared to be effective in the treatment of RA patients.<sup>9–11</sup> These cytokines form a network, inducing IL-1, IL-6 and chemokines by TNF $\alpha$ , and IL-6 and chemokines by IL-1.<sup>5,6,8</sup> Macrophage-lineage cells infiltrating synovial tissues produce IL-1 $\beta$  and TNF $\alpha$ , and fibroblast-like synovial cells produce IL-1 $\alpha$ , IL-1 $\beta$  and IL-6.<sup>12</sup>

RA occurs more frequently in women as is also true of other autoimmune diseases; the ratio of RA incidence in women to men is about 2–4:1.<sup>13</sup> Serum concentrations of dehydroepiandrosterone (DHEA) and estrone concentrations

were lower and estradiol was higher in RA male patients compared with healthy controls, and estradiol correlated with the inflammation.<sup>14</sup> The levels of estrogen to androgen in synovial fluid are elevated in both male and female RA patients,<sup>15</sup> and the level of testosterone in synovial fluid is low in male RA patients as compared to normal individuals.<sup>16</sup> In addition, women have a more severe disease with a considerably lower remission rate than men.<sup>17</sup> Therefore, sex hormones are implicated in the gender difference in both the onset and progression of RA.

It is reported that synovial macrophages from RA patients metabolize testosterone to the bioactive metabolite 5 $\alpha$ -dihydrotestosterone (DHT), and testosterone inhibits IL-1 $\beta$  production from the cells.<sup>18</sup> In contrast, the effects of androgen on the cytokine production from fibroblast-like synovial cells are largely unknown. We have reported that 17 beta-estradiol (E<sub>2</sub>) induced IL-1 $\alpha$  mRNA expression,<sup>19</sup> whereas DHT inhibited TNF $\alpha$ -induced IL-1 $\alpha$  mRNA expression in RA patient-derived fibroblast-like synovial cell line MH7A.<sup>20</sup> In this study, we examined the effect of DHT on induction of IL-8, IL-6 and IL-1 $\beta$  by IL-1 $\alpha$  or TNF $\alpha$  in MH7A cells, and suggested that DHA inhibits the effects of IL-1 $\alpha$  and TNF $\alpha$  by inhibiting nuclear factor (NF)- $\kappa$ B activation in a manner dependent on the androgen receptor (AR).

### MATERIALS AND METHODS

**Reagents** Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); fetal bovine serum (FBS) was from JRH Bioscience (Lenexa, KS, U.S.A.); 5 $\alpha$ -dihydrotestosterone (DHT) was from Fluka (Buchs, Switzerland). Human recombinant IL-1 $\alpha$  (2 $\times$ 10<sup>7</sup> U/mg based on the thymocyte co-mito-

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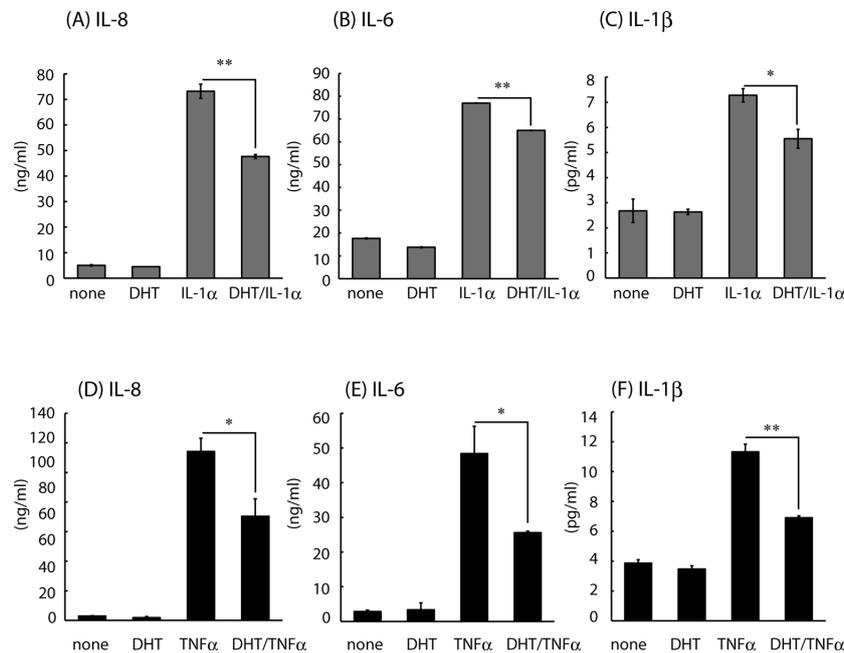


Fig. 1. Effect of DHT on the IL-1 $\alpha$  or TNF $\alpha$ -Induced Production of Proinflammatory Cytokines from MH7A Cells

MH7A cells were pretreated with DHT (10 nM) for 2 h and treated for another 24 h with IL-1 $\alpha$  (1000 U/ml). For treatment with TNF $\alpha$  the cells were transiently transfected with pcDNA-F-AR. After 16 h, cells were pretreated with DHT (10 nM) for 2 h and treated for another 24 h with TNF $\alpha$  (10 U/ml). Then culture supernatants were obtained, and IL-8 (A, D), IL-6 (B, E), IL-1 $\beta$  (C, F) protein levels were determined by enzyme-linked immunosorbent assay (ELISA). \*\* $p < 0.01$ , \* $p < 0.05$ , between IL-1 $\alpha$  and DHT/IL-1 $\alpha$  or TNF $\alpha$  and DHT/TNF $\alpha$ . Means and standard deviations (error bar) based on triplicates are shown.

genic activity) and human recombinant TNF $\alpha$  ( $6 \times 10^7$  U/mg based on the cytotoxic activity against L929 cells cultured with actinomycin D) were provided by Dainippon Pharmaceutical Co. (Osaka, Japan).

**Cell Culture** MH7A cells, an immortalized cell line established by stably transfecting rheumatoid synoviocytes with the SV40 T antigen gene<sup>21</sup> were cultured in DMEM with 100 U/ml of penicillin G, 100  $\mu$ g/ml of streptomycin, 4 mM L-glutamine and 10% heat-inactivated FBS at 37 °C in air containing 5% CO<sub>2</sub>. The day before the experiment, the culture medium was replaced by phenol red-free DMEM supplemented with 10% heat-inactivated FBS that had been pretreated with dextran-coated charcoal to remove endogenous androgen.

**Measurement of Cytokine Level** Protein levels of IL-1 $\beta$ , IL-6 and IL-8 were determined by enzyme-linked immunosorbent assays (ELISA) kit according to the manufacturer's protocol using an Endogen Human IL-1 $\beta$  ELISA Set, human IL-6 ELISA Set and human IL-8 ELISA Set (BD Biosciences, U.S.A.).

**Plasmids** pGL3-kBwt and NF- $\kappa$ B mutant construct pGL3-kBm (for NF- $\kappa$ B reporter gene assay) were kindly provided by Dr. T. Okamoto (Nagoya City University Graduate School of Medical Sciences). pcDNA3-F-AR (AR expression vector) was kindly provided by Dr. A. Ariga (Faculty of Pharmaceutical Sciences, Hokkaido University). pGL3-IL-8 (WT) and pGL3-IL-8 (mutant NF- $\kappa$ B) were generously furnished by Dr. N. Mukaida (Cancer Research Institute, Kanazawa University). pGL4-activator protein (AP)<sub>1</sub>-Luc, pGL4-CCAAT-enhancer-binding protein (C/EBP) (C/EBP)<sub>6</sub>-Luc, pGL4(NF- $\kappa$ B-like)<sub>6</sub>-Luc and pGL4-(Ig- $\kappa$ B)<sub>6</sub>-Luc were generated by polymerase chain reaction (PCR).

**Real Time PCR Analysis** Assays were performed using

an Applied Biosystems 7300 sequence detector (Applied Biosystems, Foster City, CA, U.S.A.) as described previously.<sup>22</sup> For the analysis of human IL-1 $\beta$ , IL-6 and IL-8, each amplification mixture (20  $\mu$ l) was made to contain 80 ng cDNA, 10  $\mu$ l Premix EX Taq<sup>TM</sup> (pPerfect Real Time) (RR039A) (Takara Bio, Japan), 1  $\mu$ l PCR primer (forward and reverse primer), 0.4 ml Rox Reference Dye (Takara Bio), and distilled water. The reaction mixtures were incubated at 95 °C for 10 s, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and then cooled to 4 °C. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the reaction mixture (20  $\mu$ l) contained 40 ng cDNA, 10  $\mu$ l POWER SYBER Green PCR Master Mix (P/N 4367659) (Applied Biosystems), 2  $\mu$ l PCR primer (forward and reverse primers) (5  $\mu$ M), and distilled water. The reaction mixtures were incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 58.2 °C for 1 min, 60 °C for 30 s, 95 °C for 15 s, and then cooled to 4 °C. Data were analyzed with the ABI Sequence Detector's software. The results were expressed as mean  $\pm$  S.D. for the relative expression levels compared with GAPDH, and minimum values of four independent experiments were used. The TaqMac probes and primers for IL-1 $\beta$  (Hs00174097 m1), IL-6 (Hs00174131 m1) and IL-8 (Hs00174103 m1) were obtained from Applied Biosystems. Primers for human GAPDH, forward primer 5'-cctgcaccaccaactgctta-3' and reverse primer, 5'-TCTTCTGGGTGGCAGTGATG-3', were obtained from the Japan Genetic Institute (Sendai, Japan).

**Transient Transfection and Luciferase Assays** The day before transient transfection of MH7A cells, the culture medium was replaced by phenol red-free DMEM supplemented with 10% heat-inactivated FBS that had been pretreated with dextran-coated charcoal to remove endogenous sex hormones. Reporter plasmid, AR expression plasmid and pCMV- $\beta$  gal plasmid (for normalization of transfection effi-

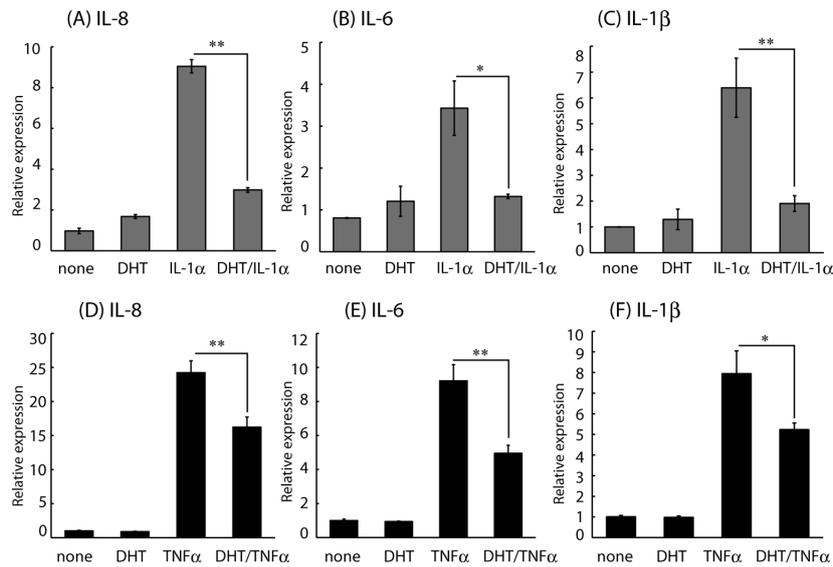


Fig. 2. Effect of DHT on the mRNA Expression of IL-1α or TNFα-Induced Proinflammatory Cytokines from MH7A Cells

MH7A cells were pretreated with DHT (10 nM) for 2 h and treated for another 24 h with IL-1α (1000 U/ml). For treatment with TNFα the cells were transiently transfected with pcDNA-F-AR. After 16 h, cells were pretreated with DHT (10 nM) for 2 h and treated for another 24 h with TNFα (10 U/ml). Then total RNA was extracted, and IL-8 (A, D), IL-6 (B, E), IL-1β (C, F) and GAPDH mRNA expression levels were determined by real-time PCR. \*\**p*<0.01, \**p*<0.05, between IL-1α and DHT/IL-1α or TNFα and DHT/TNFα. Means and standard deviations (error bar) based on triplicate levels are shown.

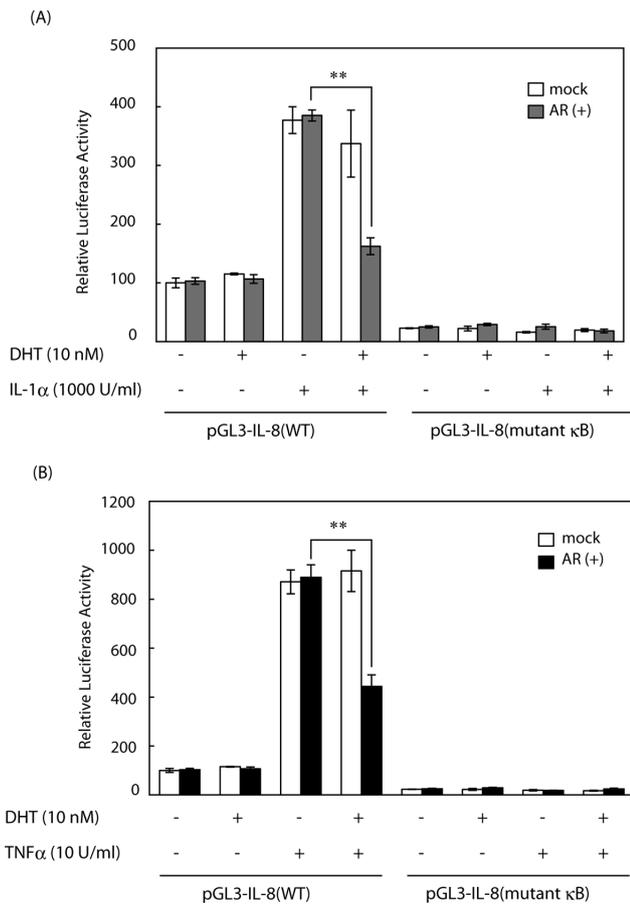


Fig. 3. Effect of DHT on the IL-1α or TNFα-Induced Promoter Activity of the IL-8 Gene in MH7A Cells

MH7A cells were transiently transfected with pcDNA3-F-AR, pGL3-IL-8 (WT) or pGL3-IL-8 (mutant κB) and pCMV-β-gal. After 16 h, cells were treated with or without DHT (10 nM) in the presence or absence of IL-1α (1000 U/ml) or TNFα (10 U/ml) for 24 h and luciferase activity was measured. The luciferase activity was normalized by β-galactosidase activity and expressed as a percentage of control (untreated cells with pGL3-IL-8 (WT) transfection). \*\**p*<0.01, between IL-1α-AR and DHT/IL-1α-AR or TNFα and DHT/TNFα-AR. Means and standard deviations (error bar) based on triplicate are shown.

ciency) were transiently transfected into MH7A cells using the calcium phosphate-DNA co-precipitation method. After 16 h of transfection, cells were incubated with DHT for an additional 24 h and harvested. Luciferase assays were performed with the luciferase reporter gene assay kit (Roche, Germany) according to the manufacturer's instructions. The light emission was measured using a multilabel counter 1420 ARVO (Perkin Elmer, Wellesley, MA, U.S.A.). Luciferase activity was expressed after normalization with the β-galactosidase value in the same sample.

**Western Blot Assay** Cells were lysed in a buffer composed of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton-X, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride for, 10 μg/ml leupeptin and 50 μg/ml aprotinin by incubating for 30 min at 4 °C. The proteins were separated by 8% SDS-polyacrylamide gel electrophoresis (PAGE) and were transferred to a polyvinylidene difluoride (PVDF) microporous membrane, Immobilon TMPVDF (Millipore, Bedford, MA, U.S.A.). After blocking with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20, membranes were incubated with anti-IκBα (c-15) rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.), anti-β-actin (AC-15) mouse mAb (Sigma Chemical Co.) or anti-Flag M2 mouse monoclonal antibody (Sigma Chemical Co.), and then with horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin (Ig) G (Jackson ImmunoResearch, West Grove, PA, U.S.A.) or anti-mouse Ig HRP-linked whole antibody (GE Healthcare U.K., Ltd.). The reactive proteins were detected with enhanced chemiluminescence reagents (GE Healthcare U.K., Ltd.) and were analyzed by a chemiluminescence image analyzer, LAS-1000 (GE Healthcare U.K., Ltd.).

**Statistic Analysis** Statistical significance between two groups was examined using unpaired Student's *t*-test. The symbol \* and \*\* indicates statistical significance at *p* values of 0.05 and 0.01 in figures, respectively.

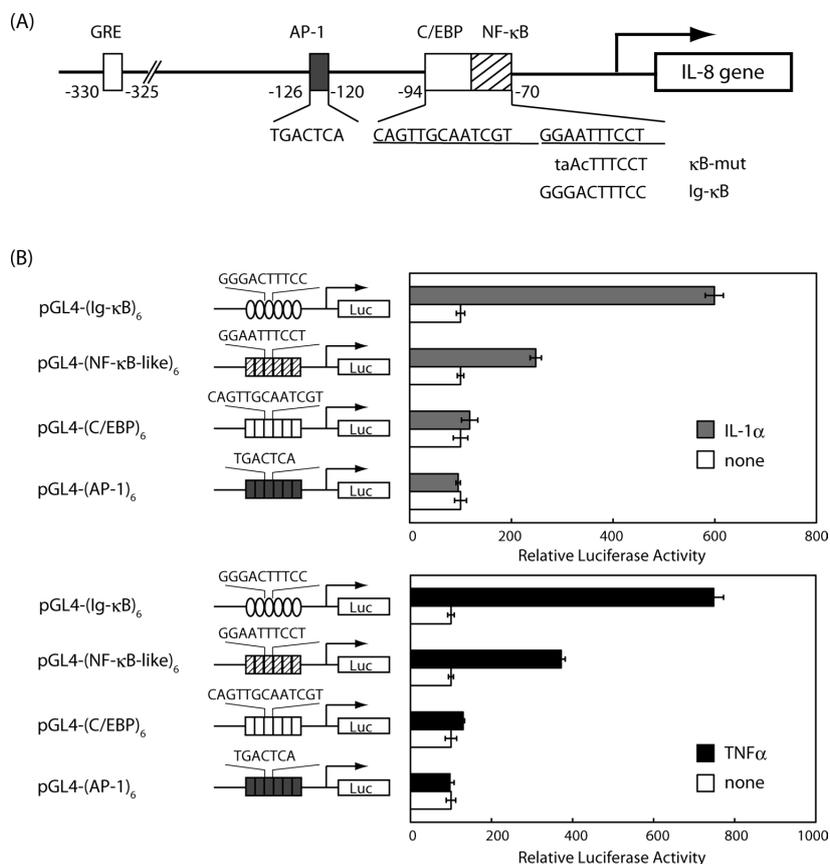


Fig. 4. Effect of DHT on the IL-1 $\alpha$  or TNF $\alpha$ -Induced pGL4-(Ig- $\kappa$ B)<sub>6</sub>-Luc, pGL4-(NF- $\kappa$ B-Like)<sub>6</sub>-Luc, pGL4-(C/EBP)<sub>6</sub>-Luc or pGL4-(AP-1)<sub>6</sub>-Luc Activity in MH7A Cells

(A) Schematic structure of the 5'-flanking region in the *IL-8* gene is shown. (B) Effect of IL-1 $\alpha$  or TNF $\alpha$  on the pGL4-(Ig- $\kappa$ B)<sub>6</sub>-Luc, pGL4-(NF- $\kappa$ B-like)<sub>6</sub>-Luc, pGL4-(C/EBP)<sub>6</sub>-Luc or pGL4-(AP-1)<sub>6</sub>-Luc activity in MH7A cells. MH7A cells were transiently transfected with pCMV- $\beta$ -gal with or without pGL4-(Ig- $\kappa$ B)<sub>6</sub>-Luc, pGL4-(NF- $\kappa$ B-like)<sub>6</sub>-Luc, pGL4-(C/EBP)<sub>6</sub>-Luc or pGL4-(AP-1)<sub>6</sub>-Luc. After 16 h, cells were treated with or without IL-1 $\alpha$  (1000 U/ml) or TNF $\alpha$  (10 U/ml) for 24 h and luciferase activity was measured. Luciferase activity was normalized by  $\beta$ -galactosidase activity and expressed as a percentage of control (untreated cells).

## RESULTS

### DHT Inhibits the IL-1 $\alpha$ or TNF $\alpha$ -Induced Production of IL-8, IL-6 and IL-1 $\beta$ from MH7A Synoviocytes

To investigate the effects of IL-1 $\alpha$  and DHT on the production of IL-8, IL-6 and IL-1 $\beta$  from MH7A cells, the cells were treated with or without IL-1 $\alpha$  in the presence or absence of a physiological concentration of DHT; serum level of total testosterone is  $20.10 \pm 4.50$  and  $19.10 \pm 4.50$  nM in normal men and RA men, respectively. In women, serum testosterone is  $0.78 \pm 0.27$  and  $0.20 \pm 0.06$  nM in normal individuals and RA patients, respectively.<sup>23)</sup> As shown in Figs. 1A–C, IL-1 $\alpha$  induced IL-8, IL-6 and IL-1 $\beta$  production from the cells, and DHT significantly inhibited the effect of IL-1 $\alpha$ . DHT alone did not influence the level of these cytokines. In contrast to the effect on IL-1 $\alpha$ , DHT did not significantly inhibit the TNF $\alpha$ -induced IL-8, IL-6 and IL-1 $\beta$  production (data not shown). However, in the cells overexpressed with AR, DHT did exhibit the inhibitory effect (Figs. 1D–F).

### DHT Inhibits IL-1 $\alpha$ or TNF $\alpha$ -Induced IL-8, IL-6 and IL-1 $\beta$ mRNA Expression

In order to determine whether the inhibitory effect of DHT was at the level of mRNA of these cytokines, we determined the effects of DHT on the mRNA expression of IL-8, IL-6 and IL-1 $\beta$  induced by IL-1 $\alpha$  or TNF $\alpha$ . As shown in Figs. 2A–C, DHT significantly inhibited the IL-1 $\alpha$ -induced mRNA expression of IL-8, IL-6

and IL-1 $\beta$ . It did not significantly inhibit the effect of TNF $\alpha$  on MH7A cells (data not shown), while it did inhibit the TNF $\alpha$ -induced mRNA expression of IL-8, IL-6 and IL-1 $\beta$  in the cells overexpressed with AR (Figs. 2D–F). Similar inhibitory effects of DHT against IL-1 $\alpha$  and TNF $\alpha$  were observed at an early incubation time of 8 h (data not shown).

**DHT Inhibits the IL-1 $\alpha$  or TNF $\alpha$ -Induced Promoter Activity of the *IL-8* Gene via AR** We determined whether or not DHT inhibits the effects of IL-1 or TNF $\alpha$  at the level of promoter activity by employing reporter plasmids of the *IL-8* gene. MH7A cells were transiently transfected with reporter plasmids of the *IL-8* gene with or without the expression vector of AR, then the cells were treated with or without DHT in the presence or absence of IL-1 $\alpha$  or TNF $\alpha$  for 24 h, and luciferase activity was measured. As shown in Fig. 3, IL-1 $\alpha$  or TNF $\alpha$  induced transactivation of the *IL-8* gene. DHT alone did not inhibit the effect of IL-1 $\alpha$  or TNF $\alpha$  without AR overexpression. However, it did significantly inhibit IL-1 $\alpha$  or TNF $\alpha$ -induced transactivation of the *IL-8* gene in the cells overexpressed with AR.

**NF- $\kappa$ B Activation Is Critical for the IL-1 $\alpha$  or TNF $\alpha$ -Induced Transactivation of the *IL-8* Gene in MH7A Cells** As shown in Fig. 3, neither IL-1 $\alpha$  nor TNF $\alpha$  induced transactivation of the *IL-8* gene with mutant NF- $\kappa$ B binding site, indicating that NF- $\kappa$ B is critical for the activation of the *IL-8* gene. It has been reported that the *IL-8* gene activation is reg-

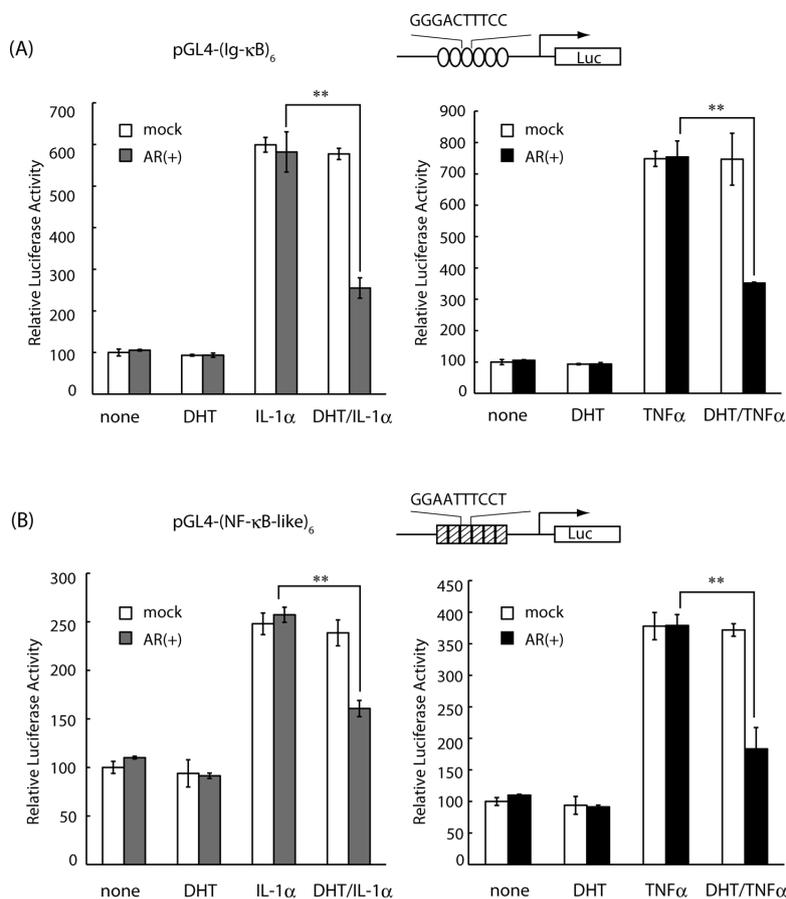


Fig. 5. Effect of DHT on the IL-1 $\alpha$  or TNF $\alpha$ -Induced Activity of pGL4-(Ig- $\kappa$ B)<sub>6</sub>-Luc or pGL4-(NF- $\kappa$ B)<sub>6</sub>-Luc in MH7A Cells

MH7A cells were transiently transfected with pCMV- $\beta$ -gal with or without pGL4-(Ig- $\kappa$ B)<sub>6</sub>-Luc, pGL4-(NF- $\kappa$ B-like)<sub>6</sub>-Luc or pcDNA3-F-AR. After 16 h, cells were treated with or without IL-1 $\alpha$  (1000 U/ml) or TNF $\alpha$  (10 U/ml) in the presence or absence of DHT (10 nM) for 24 h and luciferase activity was measured. (A) Effect of DHT on the IL-1 $\alpha$  or TNF $\alpha$ -induced pGL4-(Ig- $\kappa$ B)<sub>6</sub>-Luc activity. (B) Effect of DHT on the IL-1 $\alpha$  or TNF $\alpha$ -induced pGL4-(NF- $\kappa$ B-like)<sub>6</sub>-Luc activity. Luciferase activity was normalized by  $\beta$ -galactosidase activity and expressed as a percentage of control (untreated cells with empty vector transfection). \*\* $p < 0.01$ , between IL-1 $\alpha$ -AR and DHT/IL-1 $\alpha$ -AR or TNF $\alpha$  and DHT/TNF $\alpha$ -AR. Means and standard deviations (error bar) based on triplicate are shown.

ulated by AP-1 or C/EBP as well, depending on cell type.<sup>24</sup> In order to confirm the critical role of NF- $\kappa$ B activation for the IL-1 $\alpha$  or TNF $\alpha$ -induced transactivation of the *IL-8* gene in MH7A cells, we performed the reporter gene assay using cells transfected with pGL4-(Ig- $\kappa$ B)<sub>6</sub>, pGL4-(NF- $\kappa$ B-like)<sub>6</sub>, pGL4-(C/EBP)<sub>6</sub> or pGL4-(AP-1)<sub>6</sub>. Each element except for Ig- $\kappa$ B was derived from the *IL-8* promoter gene. As shown in Fig. 4, luciferase activities from pGL4-(Ig- $\kappa$ B)<sub>6</sub> or pGL4-(NF- $\kappa$ B-like)<sub>6</sub> were markedly augmented by IL-1 $\alpha$  or TNF $\alpha$  treatment, while those from pGL4-(C/EBP)<sub>6</sub> or pGL4-(AP-1)<sub>6</sub> were not at all or only slightly activated, indicating the importance of NF- $\kappa$ B activation in MH7A cells.

**DHT Inhibits the IL-1 $\alpha$  or TNF $\alpha$ -Induced NF- $\kappa$ B Activation via AR** In order to determine whether the inhibition of IL-1 $\alpha$  or TNF $\alpha$ -induced NF- $\kappa$ B activation by DHA is dependent on AR, MH7A cells were transfected with the reporter plasmid for NF- $\kappa$ B with or without overexpression of AR, and then treated with or without IL-1 $\alpha$  or TNF $\alpha$  in the presence or absence of DHT. As shown in Fig. 5, IL-1 $\alpha$  or TNF $\alpha$  augmented luciferase activity from pGL4-(Ig- $\kappa$ B)<sub>6</sub> or pGL4-(NF- $\kappa$ B-like)<sub>6</sub>. DHT did not inhibit the IL-1 $\alpha$  or TNF $\alpha$ -induced luciferase activity. However, in the cells overexpressed with AR, DHT did exhibit the inhibitory effect.

**DHT Inhibits the IL-1 $\alpha$  or TNF $\alpha$ -Induced I $\kappa$ B Degradation** I $\kappa$ B degradation is a critical step for the activation

of NF- $\kappa$ B. In order to determine whether DHT inhibits the IL-1 $\alpha$  or TNF $\alpha$ -induced NF- $\kappa$ B activation by inhibiting I $\kappa$ B degradation, MH7A cells were treated with or without IL-1 $\alpha$  or TNF $\alpha$  in the presence or absence of DHT, and then I $\kappa$ B expression level was determined. As shown in Fig. 6, IL-1 $\alpha$  or TNF $\alpha$  induced degradation of I $\kappa$ B. DHT significantly reversed the effect of IL-1 $\alpha$  (Fig. 6A). In contrast, this reversal effect was not observed in the cells without overexpression of AR (Fig. 4B), but was observed in those overexpressed with AR (Fig. 6C).

## DISCUSSION

In this study we first showed that DHT inhibits IL-1 $\alpha$  or TNF $\alpha$ -induced production of IL-1 $\beta$ , IL-6 and IL-8 from MH7A synoviocytes. The inhibitory effect of DHT was at the level of mRNA expression of these cytokines. We further demonstrated that DHT inhibits IL-1 $\alpha$  or TNF $\alpha$ -induced promoter activity of the *IL-8* gene, which was dependent on AR. It is reported that the *IL-8* gene activation is regulated by NF- $\kappa$ B, C/EBP and AP-1, each of them alone or in synergy, depending on cell type.<sup>23</sup> In MH7A cells the NF- $\kappa$ B binding site in the promoter region of the *IL-8* gene appeared to be critical for the IL-1 $\alpha$  or TNF $\alpha$ -induced transactivation of the gene. This was confirmed by the observation that NF- $\kappa$ B, ei-

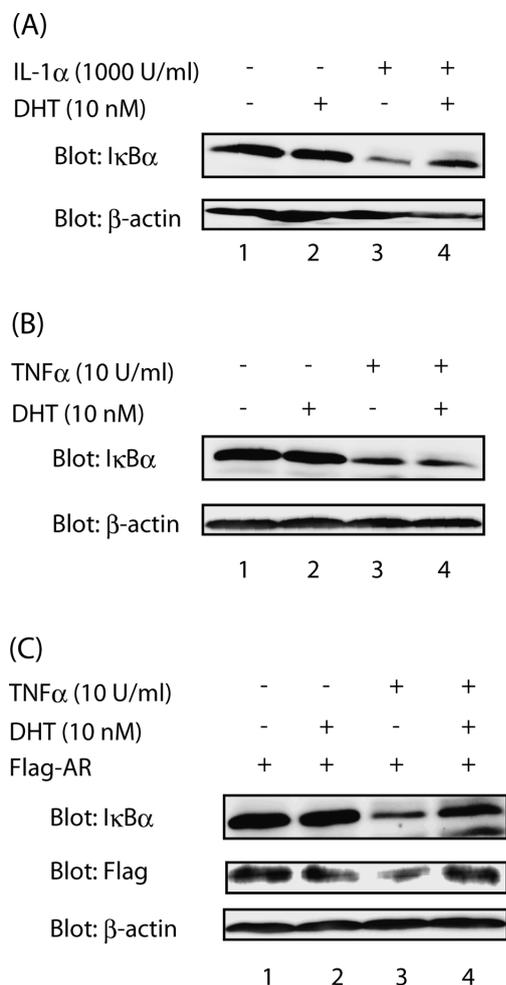


Fig. 6. Effect of DHT on the IL-1 $\alpha$  or TNF $\alpha$ -Induced I $\kappa$ B $\alpha$  Degradation

MH7A cells were transiently transfected without (A, B) or with (C) pcDNA3-F-AR. After 16h, cells were treated with or without IL-1 $\alpha$  (1000 U/ml) (A) or TNF $\alpha$  (10 U/ml) (B, C) for 30 min in the presence or absence of DHT (10 nM). Cell extracts were obtained and protein levels of I $\kappa$ B $\alpha$ , AR-FLAG and  $\beta$ -actin were determined by Western blotting with antibodies against I $\kappa$ B $\alpha$ , FLAG or  $\beta$ -actin. The lower molecular weight band of (C), lane 4 Blot-I $\kappa$ B $\alpha$  was nonspecific. Representative data of three independent experiments are shown.

ther IL-8 gene- or Ig-kB-derived, was markedly activated by IL-1 $\alpha$  or TNF $\alpha$ , while C/EBP and AP-1 were not at all or only slightly activated. In addition, DHT inhibited NF- $\kappa$ B activation as demonstrated by the inhibition of IL-1 $\alpha$  or TNF $\alpha$ -induced activation of the reporter gene with NF- $\kappa$ B in a manner dependent on AR. In these experiments DHT inhibited the IL-1 $\alpha$ -induced production and mRNA expression of IL-1 $\beta$ , IL-6 and IL-8 without overexpression of AR. The IL-1 $\alpha$ -dependent degradation of I $\kappa$ B, a critical step for NF- $\kappa$ B activation, was also inhibited by DHT without overexpression of AR. However, such overexpression was necessary for the inhibitory effects of DHT on the IL-1 $\alpha$ -induced promoter activation of the IL-8 gene. Therefore, the expression level of endogenous AR alone is not sufficient for DHT to exert its maximum effect. In contrast to IL-1 $\alpha$ , the inhibitory effect of DHT on all the effects of TNF $\alpha$  required overexpression of AR. It is probable that signals from TNF $\alpha$  are stronger than those from IL-1 $\alpha$  in the cells.

AR is a member of a group of four closely related steroid receptors, the other members of which are glucocorticoid receptor, mineralocorticoid receptor, and progesterone recep-

tor.<sup>25)</sup> AR consists of four domains, an N-terminal domain that is involved in transcriptional regulation, a DNA-binding domain, a hinge region and a C-terminal ligand-binding domain. The ligand binds to AR in the cytoplasm, and then the ligand-activated AR forms a homodimer, translocates into nucleus, binds to target DNA, interacts with coactivators and induces transcription of the genes. The mechanism by which DHT/AR inhibits NF- $\kappa$ B activation is not yet known. In COS-1 cells overexpression of RelA (p65) repressed AR-mediated transactivation with weak interaction between AR and RelA,<sup>26)</sup> and in prostate cancer LNCaP cells DHT suppressed NF- $\kappa$ B activity accompanying a slight increase of I $\kappa$ B level.<sup>27)</sup> We showed that DHT inhibits IL-1 $\alpha$  or TNF $\alpha$ -induced I $\kappa$ B degradation. However, the precise mechanism remains to be elucidated.

It is reported that mRNA and protein of AR are expressed in the synovium tissue. Relative abundance of AR mRNA was significantly lower in RA than in non-inflamed synovial tissue, and the expression level of AR protein was significantly higher in lining cells and fibroblasts than that in inflammatory cells.<sup>28)</sup> It is also reported that AR is expressed in synoviocytes, and that DHT inhibits the IL-1 $\beta$ -induced production of IL-6.<sup>29)</sup> Therefore, it is possible that DHT has a preventive effect against the onset and/or development of RA, which is dependent on the expression of functional AR in the synovium tissue.

IL-8 is a chemokine for neutrophils and T cells, which is strongly induced by IL-1 $\alpha$ , IL-1 $\beta$  or TNF $\alpha$ . The pathogenic role of IL-8 in RA was suggested by the observation that as the disease worsens and the cell count infiltrating into synovium increases, the polymorphonuclear leucocyte becomes the predominant cell type.<sup>30)</sup> The IL-8 level was higher in synovial fluid from RA patients than in synovial fluid from osteoarthritis patients or from patients with other arthritides. Synovial tissue fibroblasts release only small amounts of constitutive IL-8 but could be induced to produce IL-8 by stimulation with either IL-1 $\beta$  or TNF $\alpha$ ,<sup>31)</sup> which is in agreement with our study. We also showed that DHT inhibits IL-1 $\alpha$  or TNF $\alpha$ -induced production and mRNA of IL-1 $\beta$  and IL-6. NF- $\kappa$ B activation is critical for induction of these cytokines. Along with TNF $\alpha$ , IL-1 $\beta$  and IL-6 are major players in the onset and development of RA.<sup>5-7)</sup> Therefore, our study suggests that DHT is a negative regulator of the pathogenesis of RA by inhibiting TNF $\alpha$  or IL-1-induced production of proinflammatory cytokines from synovial fibroblast-like cells, thereby contributing to the gender differences of RA.

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