17 α -Hydroxylase/C17-20 Lyase Cytochrome P450 mRNA Expressions and Enzyme Activities during the Development of Arthritis in Collagen-Induced Arthritis Mice

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In our previous study, we have investigated the serum levels of dehydroepiandrosterone (DHEA) in type II collagen (CII)-induced arthritis (CIA) mice. During the study, we found that in normal control mice, serum levels of DHEA in the latter half of the experimental period (13–16 weeks old) were significantly lower than those at the beginning of the experiment (10 weeks old). However, in CIA mice, such decreases were not observed by CII treatment. To examine the cause of the retention of DHEA during the development of arthritis in CIA mice in this study, 17 α -hydroxylase/C17-20 lyase P450 (CYP17) mRNA expressions were measured by real time RT-PCR and the CYP17 enzyme activities were investigated in the liver and testis on days 6, 13, 28 and 48 after CII treatment in DBA/1J mice. There were no significant differences of CYP17 expressions in the liver between control and CIA mice at each experimental day, while a significant increase of expression in the testis of CIA mice was observed on day 48. On the other hand, CYP17 enzyme activities on days 28 and 48 in testis microsome (Mc) from the CIA mice were significantly higher than those of the control on the same day, while no significant differences of activities in liver Mc were observed between the CIA and control mice. These findings suggested that the cause of the retention of DHEA on days 28 and 48 after CII treatment may be the increase of CYP17 expression and the enzyme activities in the testis.

Key words rheumatoid arthritis; collagen-induced arthritis; dehydroepiandrosterone; CYP17 mRNA

A large number of clinical and experimental studies provide evidence that a low serum level of dehydroepiandrosterone (DHEA) is associated with a number of inflammatory diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE).^{1–3} DHEA has also been shown to have therapeutic effects in the treatment of RA^{4–6} and SLE.^{2,7,8} The biochemical and molecular mechanisms of action of DHEA, however, are not known.

In our previous study, we examined the participation of endogenous DHEA in the pathology of the onset of collagen-induced arthritis (CIA) in DBA/1J male mice which are an experimental animal model for RA. During the study, we found that the serum levels of DHEA in the latter half of the experimental period (13–16 weeks old) were significantly lower than those at the beginning of the experiment (10 weeks old) in normal control mice.⁹ DHEA is synthesized from pregnenolone by 17 α -hydroxylase/C17-20 lyase cytochrome P450 (CYP17). In rodents, the CYP17 localizes in the gonads and peripheral tissues such as the liver and skin, but not in the adrenal glands.^{10,11} Katagiri *et al.* reported that CYP17 mRNA and the protein expressions in the liver were detected in 1- and 3-week-old Wistar rats but not in adult male and female rats. Further, the levels of CYP17 activities in liver microsomal fraction increased by 3 weeks of age and then were markedly reduced at 7 weeks, which is consistent with the expression levels of CYP17.¹² Vianello *et al.* also reported that the expression of hepatic CYP17 activity undergoes dramatic changes during the postnatal period, reaching very high levels by 7–8 d and disappearing before adulthood in Wistar rats.¹³ Thus the decrease of serum levels of DHEA with aging in our previous study may be a characteristic features of rodents. However, serum levels of DHEA in the lat-

ter half of the experimental period after the treatment with type II collagen (CII) were not decreased compared with those at the beginning of the CII treatment in CIA mice. We had speculated that the retention of serum DHEA levels in CIA mice may be due to the response to the protection against CIA.

In this study, to investigate the cause of the retention of DHEA during the development of arthritis in CIA mice, CYP17 mRNA expressions and the enzyme activities of CYP17 were measured in the liver and testis of the CIA mice.

MATERIALS AND METHODS

Animals Male DBA/1J mice (8 weeks old) were obtained from Charles River Japan (Kanagawa, Japan). After a resting period of 1 week, these animals were subjected to the experimental protocols.

Induction of Arthritis, and Isolation of Liver and Testis from Mice Arthritis was induced by an injection of CII as described elsewhere.⁹ Liver and testis were removed from the mice on day 6, 13, 28 and 48 after the first CII treatment. The organs from 3 mice of 6 mice were stored at –80 °C for RT-PCR analysis and the remaining organs were used for the preparation of microsomal fractions immediately after the isolation.

Preparation of Microsomes (Mc) from Liver and Testis Liver and testis obtained from 3 mice of each group were combined. The tissues were minced and homogenized in a loose-fitting Teflon-glass homogenizer in 4 times their weight of ice-cold 10 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose. After the homogenate was centrifuged at

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600 *g* for 10 min, the supernatant was centrifuged at 5000 *g* for 10 min, and again at 105000 *g* for 60 min. The resultant precipitate was reconstituted with 10 mM Tris-HCl buffer (pH 7.4) without sucrose to the original concentration of the homogenate and used as Mc. The protein concentrations of the Mc fractions were determined according to the dye-complex method reported by Bradford using a Protein Assay kit (Bio-Rad).¹⁴⁾ Bovine serum albumin was used as a standard. The Mc samples were stored at -80°C until use.

Real Time Quantitative RT-PCR Total RNA was extracted from the liver and testis by the phenol-chloroform-based method (ISOGEN: Nippon Gene Co. Ltd, Toyama, Japan) according to the manufacturer's instructions. cDNA synthesis from 1 μg of total RNA was carried out using a ReverTra Ace- α -cDNA Synthesis kit (Toyobo Co., Ltd, Osaka, Japan) according to the manufacturer's instructions. Real time quantitative PCR was performed using the Gene Amp 5700 Sequence Detection System (Applied Biosystems) with the following primers: CYP17, 5'-GGCTTTCCTGGTGCACAATC-3' (forward), 5'-GGAGGTGAGTCCGGT-CATTGAA-3' (reverse); β -actin, 5'-TGCCTGACATCAAGAGAAG-3' (forward), 5'-GATGCCACAGGATTCCATA-3' (reverse). CYP17 expression was analyzed with SYBR Green Mastermix (Perkin-Elmer) and a final concentration of 200 nM primers, using β -actin as the internal standard. The following PCR profile was used: 2 min at 50°C , 10 min 94°C , and 35 cycles of 30 s at 94°C , 30 s at 58°C , and 1 min at 72°C . The amount of SYBR Green was measured at the end of each cycle. The cycle number at which the emission intensity of the sample rises above the baseline is referred as to the Ct (threshold cycle) and is proportional to the target concentration. Real time PCR was performed in duplicate and analyzed by a Gene Amp 5700 Sequence Detection System manual (threshold: 0.05, baseline: 6–15 cycles). To generate the standard curves for CYP17 and β -actin, serially diluted cDNA (1/1–1/16) was prepared and real time PCR was performed as above. Standard curves were constructed by plotting the relative ratio of CYP17 gene expression level.

Measurement of CYP17 Enzyme Activity A methanol solution of pregnenolone (0.1 μmol) was added to the incubation vial and the methanol was evaporated by blown nitrogen gas. Then, 10 mM Tris-HCl buffer (pH 7.4) containing 6 mM NADPH, 70 mM sucrose, 11.5 mM NaCl and 10 mM $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ was added to the vial. After preincubation at 37°C for 5 min, the reaction was started by the addition of the Mc fraction (for liver: Mc protein concentration 500 μg ,

for testis: Mc concentration 44.4 μg). The total incubation volume was 2 ml. The incubation was carried out at 37°C for 20 min with the vial open to the air and shaken continuously and was stopped by the addition of 3 ml of CH_2Cl_2 . The mixture was shaken rapidly and centrifuged at 600 *g* for 5 min. The resulting clear organic layer was transferred to another tube and evaporated until dry. Then, 200 μl of HPLC mobile solution ($\text{H}_2\text{O}:\text{CH}_3\text{CN}=55:45$, v/v%, solvent A) was added to the tube. The concentration of 17-hydroxypregnenolone (17-OH-Preg) in the sample (10 μl) was measured with an HPLC system (NANOSPACE SI-1, Shiseido Co. Ltd., Tokyo). The steroid was separated with Capcellpak UG 120 5 μm , 250 mm $\times$ 1.5 mm I.D. column (Shiseido) at 40°C and detected at 210 nm. The column was eluted with solvent A for 15 min, followed by 20 min with solvent B ($\text{H}_2\text{O}:\text{CH}_3\text{CN}=10:90$) to wash out the substrate, pregnenolone. At 35 min, the mobile solvent was changed to solvent A for the next run. The flow rate was 1.0 ml/min. Under these conditions, 17-OH-Preg appeared at 13.4 min.

In order to validate the above assay, it was necessary to establish that the steroid formation was proportional to both the incubation time and protein concentrations. The appearances of 17-OH-Preg were linear for a period of 30 min for the liver and testis Mc, and were proportional to the protein concentrations up to 1 mg (liver) and 70 μg (testis) of Mc protein, respectively (data not shown).

Statistical Analysis Differences between two groups were statistically analyzed by the two sample *t*-test with Welch's correction. *p* values below 0.05 were considered significant.

RESULTS

Expression of CYP17 mRNA in Liver and Testis To determine the cause of the retention of serum levels of DHEA in the latter half of the experiment (on days 28 and 48 after CII treatment in CIA mice) in our previous study,⁹⁾ the expression of CYP17 mRNA in the liver and testis was measured by real time quantitative RT-PCR. The organs were removed on days 6, 13, 28 and 48 after the first CII treatment. In liver obtained on these days, there were no significant differences of CYP17 expressions between the control and CIA mice of each experimental day (Fig. 1A). On the other hand, a significant increase in the expression in testis on day 48 of CIA mice was observed compared with the control. In comparison with the CYP17 expression in testis on day 6, signifi-

Fig. 1. CYP17 mRNA Expression in the Liver (A) and Testis (B)

Gray bars show CYP17 expression levels in the control mice and black bars show those in the CIA mice. ***p* < 0.01 vs. control on the same day; ###*p* < 0.001 vs. control on day 6.

Fig. 2. CYP17 Enzyme Activity in the Liver (A) and Testis (B)

Gray bars show CYP17 expression levels in the control mice and black bars show those in the CIA mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control on the same day; ## $p < 0.01$, ### $p < 0.001$ vs. control on day 6.

cant increases were observed on days 13 and 48 in the CIA mice. However, no significant differences were seen in the controls throughout the experiment (Fig. 1B).

CYP17 Enzyme Activity in the Liver and Testis The origin of the retention of serum levels of DHEA was also confirmed by measuring the enzyme activity of CYP17 in liver and testis. From day 6 to day 48, the activity in the liver Mc decreased gradually with significant differences in both the control and CIA mice (Fig. 2A). Moreover, no significant differences of the activities in liver Mc were seen between the control and CIA mice on days 28 and 48, the days on which the retention of serum levels of DHEA in CIA mice was observed. On the other hand, in testis Mc obtained from CIA mice, the CYP17 activities on days 13, 28 and 48 were significantly higher than those on day 6. Further, in comparison with the activities between the control and CIA mice, significant increases of the activities were observed on days 28 and 48 in the testis Mc from CIA mice (Fig. 2B).

DISCUSSION

A significant increase of the CYP17 mRNA expression in testis from the CIA mice was observed on day 48 after CII treatment compared with those in testis from day 6 and day 48 of the control (Fig. 1B). Although there was no significant difference in the expression in testis obtained from the controls and CIA mice on day 28, the tendency of elevation of expression was already seen in testis from CIA mice on day 13. Further, we investigated the changes of CYP17 enzyme activities during the course of the onset of arthritis in CIA mice. The enzyme activities on days 28 and 48 in testis Mc from CIA mice were significantly higher than those of the controls (Fig. 2B). On the other hand, no significant differences of the CYP17 activities in liver Mc were observed between the CIA and control mice on days 28 and 48 (Fig. 2A). Further, CYP17 activities of testis Mc were about 100 times higher than those of liver Mc (Fig. 2). CYP17 mRNA expressions in the testis were also elevated compared with those in the liver (Fig. 1). These findings suggested that one of the causes of the retention of serum levels of DHEA on days 28 and 48 after CII treatment may have been the increase of the expression of CYP17 mRNA and the enzyme activities in the

testis. Although we currently do not know the mechanisms behind the retention of DHEA, and the increase of CYP17 expression and the enzyme activities in CIA mice on days 28 and 48, these time points coincided with the disease onset of CIA.⁹⁾

In conclusion, increase of the expression of CYP17 mRNA and the enzyme activity after CII treatment may be the result of the physiological response in protection against the onset of arthritis in CIA mice.

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