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A novel therapy for acute hepatitis utilizing dehydroepiandrosterone in the murine model of hepatitis

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Abstract

Dehydroepiandrosterone (DHEA), one of the major androgens secreted by the adrenal cortex, has been shown to have potential immunoreguratory properties. In this study, we examined the effect of DHEA in a mouse model of hepatitis. Mice were treated with DHEA and injected with concanavalin A (Con A) or lipopolysaccharide (LPS)/D-galactosamine (GalN). Cytokine expression was measured by quantitative RT-PCR and ELISA. Apoptosis was detected by the TUNEL method and by DNA fragmentation analysis. In the DHEA-treated mice, the serum levels of ALT and expression of inflammatory mediators were significantly decreased. The number of apoptotic cells was also much lower than that observed in control, untreated mouse liver tissue. There were fewer tumor necrosis factor-α (TNF-α)-induced apoptotic cells in H4IIE hepatoma cells treated with DHEA than in non-treated cells. DHEA decreased the expression levels of mRNA transcripts encoding TNF-α and iNOS. These results suggest that DHEA can reduce T-cell-mediated injury in the liver as manifest by inhibition of the expression of several inflammatory mediators and hepatocyte apoptosis. DHEA should, thus, be considered as a novel candidate for the therapy of liver injury.

Keywords: Dehydroepiandrosterone; Hepatitis; Apoptosis; Necrosis; Inflammatory mediators; T-cell

1. Introduction

In humans, hepatitis due to infection with either hepatitis B or C virus or autoimmune reactions is a major worldwide health problem. Previous studies have suggested that immune reactions against viral or endogenous self-antigens play a major role in the liver cell damage associated with both disorders [1]. Consequently, liver damage in these and other conditions can be considered immune-mediated as a result of T-cell activation. Consistent with this notion, cytotoxic lymphocytes are likely to contribute to the histological evidence of apoptosis observed in viral hepatitis. Councilman-like bodies in the liver, a histological hallmark of this disease, have been shown to be a manifestation of liver cell apoptosis [2,3]. In addition, cytokines have been suggested to play a critical role in the pathogenesis of liver injury in these diseases. It is well known that several cytokines, especially tumor necrosis factor-α (TNF-α), which is secreted from liver macrophages and Kupffer cells (KC), causes hepatic damage in clinical and experimental sepsis and endotoxemia [4,5].

T-cells appear to play a particularly important role in immune-mediated hepatitis. T-cell activation leads to the production of various cytokines, many of which are

Abbreviations: DHEA, dehydroepiandrosterone; TUNEL, TdT-mediated dUTP-biotin nick end labeling; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; ιNOS, inhibits nitric oxide synthase; COX-2, cyclo-oxygenase-2; MIF, macrophage migration inhibitory factor; KC, Kupffer cells; Con A, concanavalin A; *scid*, severe combined immunodeficiency; HCV, hepatitis C virus; ALT, alanine transaminase; HE, hematoxylin-eosin; RT-PCR, reversetranscription-polymerase chain reaction; FBS, fetal bovine serum; LPS, lipopolysaccharide; DMSO, dimethylsulfoxide; DMEM, Dulbeccos modified Eagles medium

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associated with induction of apoptosis, a major feature of T-cell-mediated liver injury, as well as direct hepatocyte injury. Concanavalin A (Con A)-induced hepatitis is a well-established experimental T-cell-mediated hepatitis model. Con A, a plant lectin, which is a non-specific T-cell mitogen, causes damage specifically to liver cells via T-cell activation [6]. Most strains of mice are exquisitely sensitive to Con A-induced hepatitis. Consistent with the T-cell basis of the hepatitis associated with this model, severe combined immunodeficiency (*scid*) and athymic nude mice are insensitive to the development of hepatitis associated with Con A administration [6,7]. Previous studies have shown that T-cells mediate injury in this model by direct cytolysis and indirectly through secretion of cytokines and inflammation mediators such as IFN-γ, TNF-α and iNOS [5,8–10]. Eradication of hepatitis C virus (HCV) is the ideal treatment for chronic HCV-associated hepatitis. Recently, combination therapy of this disorder with peginterferon alfa-2b and ribavirin has produced a sustained virological response rate of up to 50%, which represents a remarkable response to pharmacological treatment [11]. In addition to this type of anti-viral therapy, however, pharmacological treatments that inhibit T-cell-mediated hepatocyte injury are greatly needed.

Dehydroepiandrosterone (DHEA), a major adrenal androgen, is known to induce remission of hyperglycemia, increase insulin sensitivity, protect the arteries from atherosclerotic changes and may regulate the immune system through its action on T-cells [12,13]. However, the precise physiological roles of DHEA in the human endocrine system have not been elucidated. Considering that DHEA is a major adrenal hormone in humans, elucidating its physiological role is important, particularly in gastrointestinal diseases. Given that glucocorticoid, another major adrenal hormone, has a very strong anti-inflammatory effect, we hypothesized that DHEA may share this anti-inflammatory property. Hence, the aim of this study was to investigate whether DHEA plays a role in regulating T-cell-mediated liver injury. We specifically examined whether this compound, in reducing liver injury associated with T-cell activation, could reduce cytokine production and inhibit the induction of hepatocyte apoptosis.

2. Materials and methods

2.1. Animals

Fifteen-week-old female BALB/c mice were obtained from CLEA Japan Inc. Food was purchased from Oriental Yeast Co. Ltd. Mice, ranging in weight from 27 to 31 g, were divided into two groups for examination of the effect of DHEA: 40 were given DHEA and 40 served as controls. An additional group of 36 mice was used for the examination of DHEA responses at various doses: 30 were given various doses of DHEA and 6 were controls. All animals were housed in a ventilated, temperature-controlled room (23 °C) with a 12 h light:12 h dark cycle. All animals received humane care, and study protocols complied with institution guidelines.

2.2. Reagents

All reagents were purchased from Sigma Chemical Co., except where indicated.

2.3. Concanavalin A-induced hepatitis

For examination of the effect of DHEA, animals were pretreated for one week with DHEA (150 mg/kg). Control mice were given only the standard pellet diet. All animals were then given i.v. Con A (20 mg/kg, in 300 μl pyrogen-free saline [14]). Serum and liver tissue were collected at 0, 8, 20 and 30 h after Con A injection. In a separate dose–response experiment, DHEA was given for one week at 40, 75, 100, 150 or 300 mg/kg. Control animals were fed the standard pellet diet. Mice were injected with i.v. Con A (20 mg/kg, in 300 μl pyrogen-free saline). Serum and liver tissue were collected 20 h after Con A injection.

2.4. GalN and LPS injection of mice

LPS (*Escherichia coli* serotype O111:B4) and GalN were diluted in sterile endotoxin-free normal saline. Mice were injected in the midperitoneum (250 μL total volume) with LPS (2 μg) and GalN (20 mg). At 6 h after the injections, blood was obtained from the orbital vein, and serum ALT levels were measured [15,16].

2.5. Histology

Blood was taken for measurement of serum ALT levels. Liver tissue was stained with hematoxylin-eosin (HE) according to standard laboratory procedures. Apoptotic cells were detected in liver sections of mice killed 4 h after Con A injection using the Apoptosis in situ Detection Kit (TUNEL kit; Nippon Gene Co.). DNA fragmentation was analyzed with an Apoptotic DNA Ladder Kit (Roche) according to the manufacturer's instructions.

2.6. Quantitative RT-PCR for measurement of mRNA

Quantitative RT-PCR was performed using real-time TaqMan PCR (Applied Biosystems) systems. Total RNA was purified using ISOGEN reagent (Nippon Gene Co.). Total RNA was reverse transcribed into cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems). The PCR reactions contained the commercially available primer/probes and TaqMan Universal PCR Mas-

termix (Applied Biosystems). Samples were placed in 96-well plates, amplified in an automated fluorometer (ABI Prism 7700 Sequence Detection System, Applied Biosystems) and analyzed according to the manufacturer's instructions [17]. The signal for GAPDH mRNA was used to normalize for differences in RNA extractions and for different efficiencies of cDNA synthesis.

2.7. Cytokine determination by ELISA

Sandwich ELISAs for murine TNF-α were performed using TNF-α antibody-coated wells (Biosource International Inc.) according to the manufacture's instructions. Plasma TNF-α levels were measured 8 h after Con A administration.

2.8. Detection of apoptosis in H4IIE cells

H4IIE cells were cultured in DMEM (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml of penicillin–streptomycin (Gibco BRL). Cells were cultured at 37 °C until confluent, and were then trypsinized and replated at 1×10^4 cells/dish on Lab-Tek Tissue Culture Chamber Slides (Nalge Nunc). At 12 h after replating, the cells were washed twice with PBS and the medium was changed to DMEM supplemented with 10% FBS, penicillin–streptomycin and either 1 μM DHEA or 0.01% DMSO. After 3 h, cells were treated with DMEM supplemented with 10% FBS, penicillin–streptomycin, 5 ng/ml TNF-α, 0.5 μg/ml actinomycin-D, and either 1 μM DHEA or 0.01% DMSO. After 6 h, the cells were washed twice with PBS and subjected to either TUNEL analysis or a DNA fragmentation assay.

2.9. Cell culture for measurement of expression of inflammatory mediators

The mouse macrophage cell line RAW264 was cultured in DMEM (Gibco BRL) supplemented with 10% FBS (HyClone), 100 IU/ml of penicillin–streptomycin (Gibco BRL) and 0.1 mM of non-essential amino acids (NEAA) (Gibco BRL). Cells were cultured at 37 °C until confluent and were replated at 1×10^6 cells/dish on 65-mm plastic dishes. After 12 h, cells were washed twice with PBS and treated with DMEM supplemented with 10% FBS, penicillin–streptomycin, NEAA and 10 μg/ml LPS [18]. After an additional 6 h, the cells were treated with either 1 μM DHEA or 0.01% DMSO. After 18 h, total RNA was prepared.

2.10. Statistical analysis

All results are expressed as mean ± S.D. Statistical comparisons were made using Student's *t*-test or Scheffe's method after analysis of variance. A *P*-value of less than 0.05 was considered significant, and a *P*-value of less than 0.01 as highly significant.

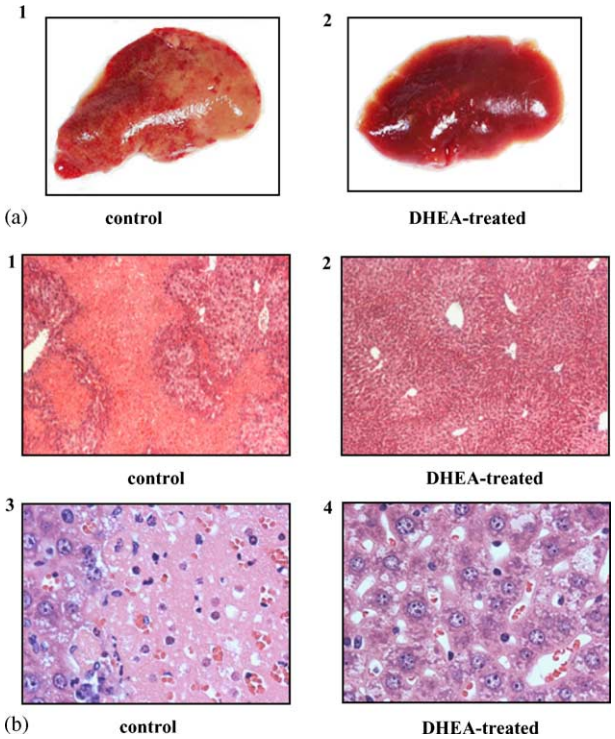

Fig. 1. Macroscopic and microscopic appearance at 30 h after Con A injection. (a) Macroscopic appearance at 30 h after Con A injection in control (1) and DHEA-treated (2) mice. Note the significant decrease in necrosis associated with DHEA treatment. (b) Hematoxylin-eosin staining of the liver from mice at 30 h after Con A injection in control (1 and 3) and DHEA-treated (2 and 4) mice. Note the significant decrease in inflammatory infiltration and maintenance of tissue architecture associated with DHEA treatment [(1 and 3), ×4; (2 and 4), ×40].

3. Results

3.1. Protective effect of DHEA on hepatitis after Con A administration

To evaluate the effect of DHEA on hepatitis, we analyzed the liver in mice at 30 h after Con A injection. Liver necrosis was widely evident in control mice in comparison to mice treated with DHEA, as revealed by macroscopic (Fig. 1a) and microscopic examination (Fig. 1b). At 30 h after Con A administration, liver damage in DHEA-treated mice was restricted to small areas of focal necrosis (Fig. 1b, panels 2 and 4) in comparison to control mice (Fig. 1b, panels 1 and 3), which exhibited widespread necrosis.

To assess the evolution of acute hepatitis, we measured serum ALT (sALT) levels at 0, 8, 20 and 30 h after Con A injection. sALT levels in DHEA-treated mice were significantly decreased in comparison with control mice (Fig. 2a). To quantify these effects of DHEA, sALT levels

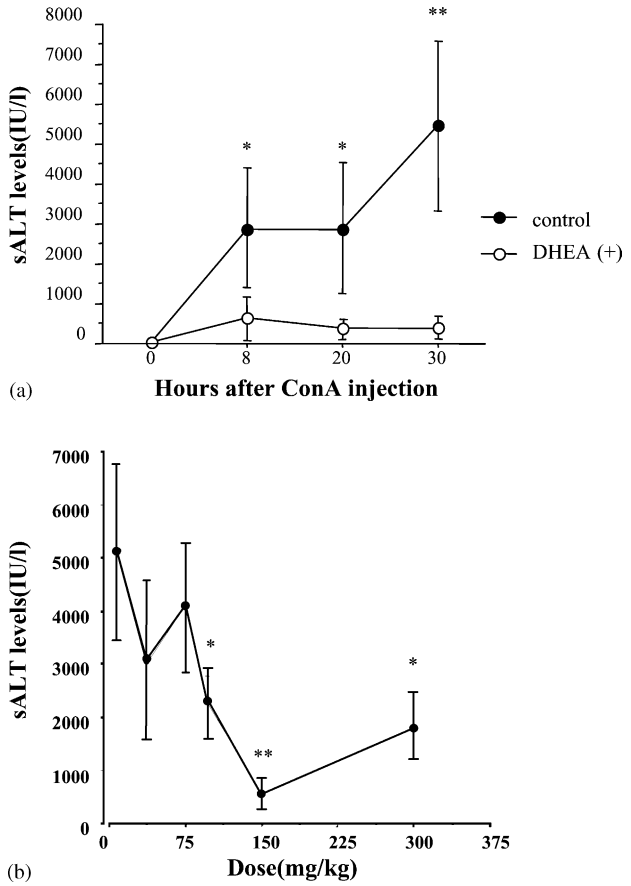

Fig. 2. Effect of DHEA in Con A-induced mice. Serum(s) ALT levels at 0, 8, 20 and 30 h after Con A injection. sALT levels were decreased in DHEA-treated mice. (B) sALT levels at various doses of DHEA treatment in mice at 30 h after ConA administration. Each point represents mean ± S.D. (*$P <$ 0.05, **$P < 0.001$).

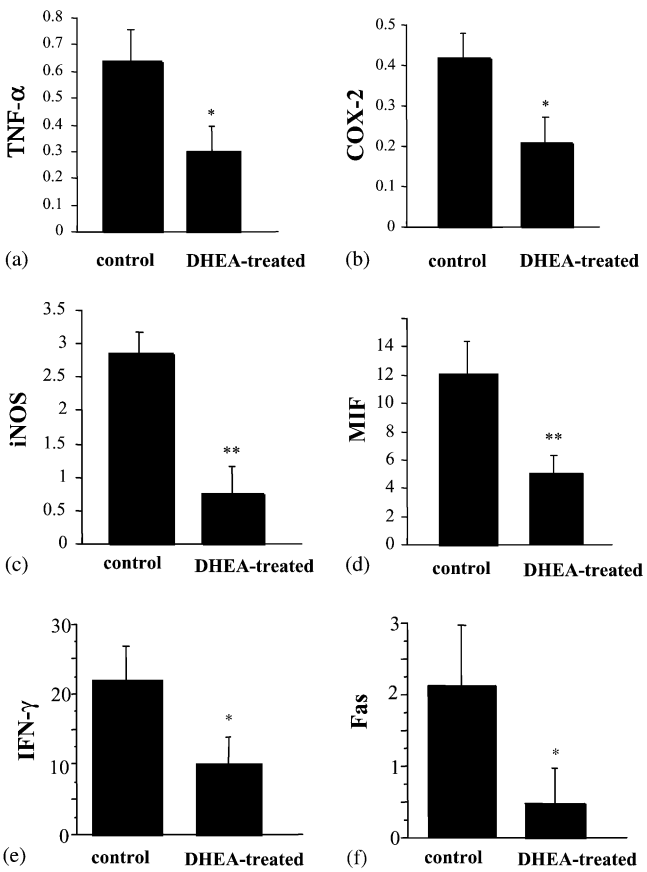

Fig. 3. Expression of inflammatory mediators in liver tissues. Quantitative analysis of real-time PCR products (a–f). The mRNA levels for all mediators were dramatically decreased in DHEA-treated mice. Each column represents mean ± S.D. (*$P < 0.05$, **$P < 0.001$).

were examined at 30 h after Con A injection at DHEA doses of 40, 75, 100, 150 and 300 mg/kg. As shown in Fig. 2b, treatment with DHEA significantly decreased sALT levels.

3.2. Modulation of inflammatory mediator expression by DHEA in the liver after Con A administration

In evaluating the effect of DHEA treatment on the development of hepatitis, we analyzed the levels of expression of mRNA encoding TNF-α, IFN-γ, iNOS, COX-2 and MIF by quantitative RT-PCR at 6 h after Con A administration. As shown in Fig. 3, the expression of IFN-γ, TNF-α and COX-2 mRNA transcripts in the liver tissue of DHEA-treated mice was significantly decreased in comparison to that of control mice. In addition, expression of iNOS and MIF mRNA transcripts was also dramatically decreased. These results indicate that DHEA suppresses the steady-state levels of mRNA transcripts encoding each of these inflammatory mediators. The level of TNF-α protein in serum was measured by ELISA and was found to be significantly suppressed by DHEA administration (Fig. 4).

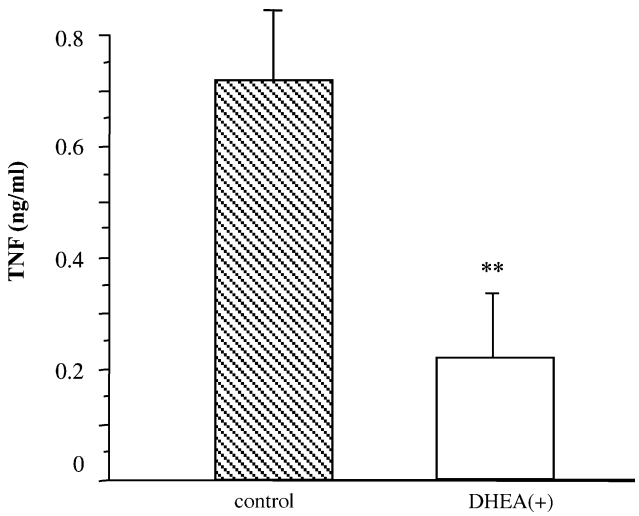

Fig. 4. Analysis of serum levels of TNF-α in Con A-injected BALB/c mice. The levels of TNF-α in the serum were determined by ELISA, 8 h after Con A injection. Vertical bars represent the mean ± S.D. Statistically significant difference compared with the control mice (**$P < 0.01$).

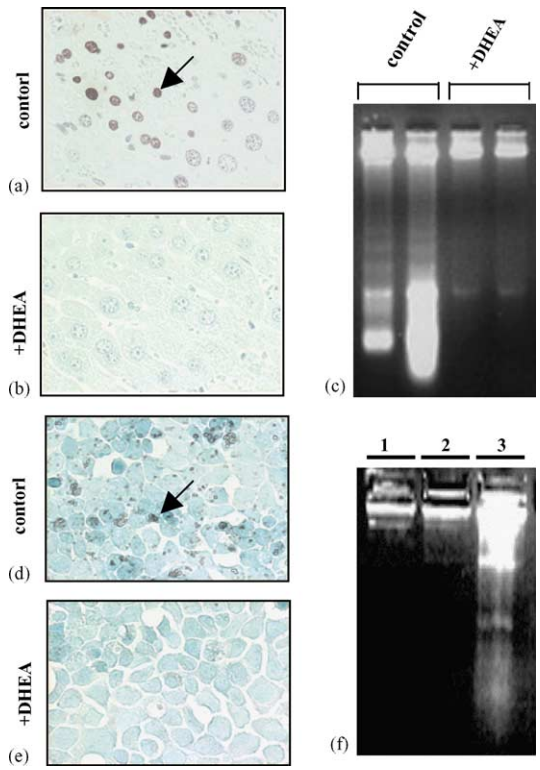

Fig. 5. Assessment of apoptosis in liver tissues and hepatocytes. TUNEL staining of liver tissues at 4 h after Con A administration (a, b). Apoptotic cells were dramatically decreased in DHEA-treated mice (×20). TUNEL staining of H4IIE cells at 4 h after treatment with TNF-α and actinomycin-D (d, e). Fewer apoptotic cells were observed in the DHEA-treated cells (×20). DNA ladder of liver tissues at 30 h after Con A administration (c). A significant decrease of DNA fragmentation was noted in DHEA-treated mice. Similar assessment of H4IIE cells (f) shows decreased DNA fragmentation with DHEA treatment (lane 1, normal cell; lane 2, DHEA-treated; lane 3, control).

3.3. Modulation of apoptosis by DHEA in liver tissue after Con A administration

Liver tissue from mice 4 h after Con A injection was examined to determine the presence and extent of apoptotic cells. As shown in Fig. 5, whereas only a few hepatocytes exhibited TUNEL-positive nuclei in sections of liver from DHEA-treated mice (Fig. 5, panel b), many TUNEL-positive hepatocytes could be detected in liver sections from control mice (Fig. 5, arrow, panel a). Furthermore, analysis of DNA fragmentation showed that whereas typical DNA fragmentation consistent with apoptosis was observed in control mouse liver, evidence of DNA fragmentation was dramatically reduced in DHEA-treated mice (Fig. 5c). Consistent with this, the expression of mRNA transcript encoding the Fas antigen was significantly reduced in liver tissue from DHEA-treated mice in comparison to non-treated mice (Fig. 3f). We conclude that apoptosis and molecules associated with apoptosis (Fas) were markedly reduced in DHEA-treated mice exposed to Con A.

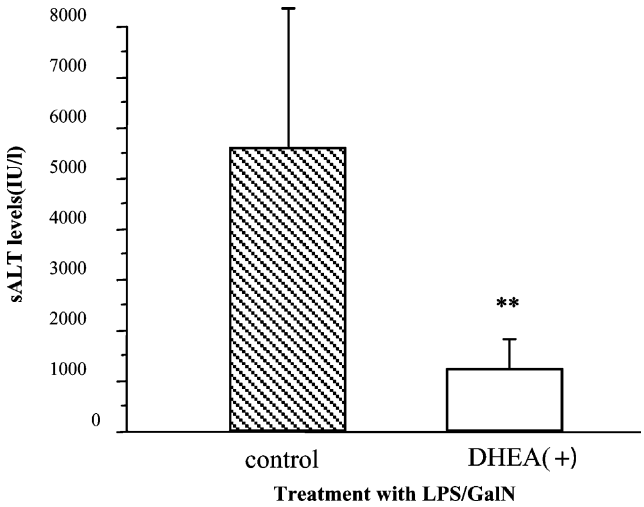

Fig. 6. Effect of DHEA in GalN/LPS models. sALT levels 6 h after i.p. injection with LPS (2 μg) and GalN (20 mg). sALT levels were decreased in DHEA-treated mice. Each point represents mean ± S.D. Statistically significant difference compared with control mice (**$P < 0.01$).

3.4. Protective effect of DHEA on hepatitis after GalN/LPS injection

To determine liver injury in response to endotoxin, we measured serum ALT levels 6 h after i.p. injection with LPS and GalN (Fig. 6). Significant differences in the degree of liver injury between DHEA-treated mice and control mice ($P < 0.01$) were observed. These results indicated that DHEA also had a protective effect against liver injury caused by LPS/GalN.

3.5. Inhibition of apoptosis by DHEA in a hepatocyte cell line

To confirm that the apoptosis described above in vivo may be related to hepatocytes, we subsequently investigated the effect of DHEA on apoptosis of H4IIE cells, a hepatocyte cell line, induced by treatment with a combination of TNF-α and actinomycin-D. As seen in panel d of Fig. 5, whereas many TUNEL-positive hepatocytes could be detected in control H4IIE cells, only a few hepatocytes exhibited TUNEL-positive nuclei in DHEA-treated cells (Fig. 5, panel e). Analysis of DNA fragmentation confirmed these results, as shown by the significant reduction in DNA fragmentation observed in the DHEA-treated cells (Fig. 5f).

3.6. Modulation of inflammatory mediator expression by DHEA in RAW264 cells

To determine whether DHEA can affect cytokine and inflammatory mediator expression by macrophages, we examined the effects of DHEA on expression of TNF-α and iNOS in LPS-treated RAW264 macrophage cells. Expression of mRNA transcript levels for TNF-α and

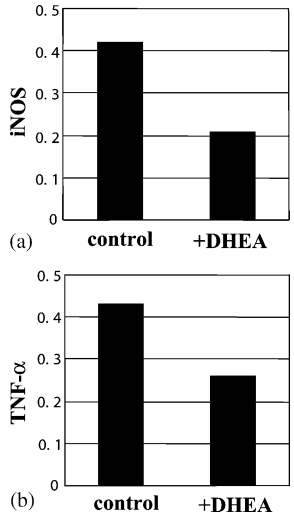

Fig. 7. Expression of iNOS and TNF-α mRNA levels in RAW264 cells. Expression of iNOS and TNF-α mRNA levels were assessed by quantitative PCR at 6 h after LPS activation of the RAW264 cell line in the presence or absence (control) of DHEA (1 μM). Note the inhibition by DHEA treatment.

iNOS were assessed by quantitative RT-PCR at 6 h after LPS exposure. As can be seen in Fig. 7, mRNA transcript levels for TNF-α and iNOS were decreased in the DHEA-treated macrophage cell line in comparison to those observed in the control, non-treated macrophage cell line.

4. Discussion

Intravenous injection of Con A is known to induce T-cell-dependent liver injury in mice [4]. It has previously been suggested that TNF-α, IFN-γ, KCs and cytotoxic T-cell activity play a central role in Con A-hepatitis [10,19]. Con A-hepatitis and viral hepatitis in humans have several similar pathologic features. These include the presence in sera of TNF-α and IFN-γ, evidence of apoptotic cell death, augmentation of Fas expression and the putative involvement of cytotoxic T-cells [8,9,17,20–23]. As such, Con A-hepatitis in mice would appear to be an appropriate model for research into the therapy of immune-mediated liver injury including that associated viral hepatitis in humans.

In this model, we demonstrated that the beneficial effect of DHEA was both related to the inhibition of inflammatory cytokines and inhibition of apoptosis in the Con A-hepatitis model. We demonstrated that DHEA dramatically reduced transaminase-release into the serum during Con A-hepatitis. Histopathological studies revealed that administration of DHEA strongly attenuated liver injury induced by Con A. With DHEA administration, liver damage was restricted to small areas of focal necrosis and the number of apoptotic cells was reduced in comparison to control mice, wherein large areas of bridging necrosis were evident in the liver. Furthermore, DHEA suppressed the levels of expression of various inflammatory mediators as defined by quantification of steady-state levels of mRNA transcripts. This was especially evident for TNF-α and IFN-γ, which are believed to induce apoptosis and necrosis in liver cells [5,8,9,20]. It has recently been reported that iNOS and COX-2 may also play a crucial role in the development of Con A-hepatitis [24]. Therefore, we analyzed mRNA expression levels for iNOS and COX-2 and found that their expression was also dramatically reduced as a consequence of DHEA administration. We used two different in vitro approaches to determine whether these effects of DHEA were independent. First, we confirmed that DHEA suppressed the levels of mRNA encoding TNF-α and iNOS in an LPS-activated macrophage cell line. Second, we observed that DHEA inhibited apoptosis induced by TNF-α in a hepatocyte cell line. Therefore, inhibition of inflammatory mediator expression by macrophages and apoptosis of liver cells are likely to be independent pharmacological effects of DHEA. These findings provide support that DHEA has a very broad pharmacological effect on T-cell-mediated hepatitis. We used the TNF-dependent GalN/LPS model [15,16] to elucidate the significance of TNF/Fas suppression by DHEA. The sALT levels in DHEA-treated mice were significantly decreased in this model. These results suggest that DHEA inhibits the TNF-α signaling pathway. The physiological role of DHEA has yet to be elucidated in the human endocrine system. However, it is known that glucocorticoid, a major adrenal hormone, ameliorates inflammation by reducing the production of inflammatory cytokines and inducing apoptosis of lymphoid cells [25,26]. Another possibility is that the physiologic role of DHEA is to minimize certain types of inflammation, such as liver injury, by both inhibiting the induction of parenchymal cell apoptosis and the production of inflammatory cytokines. The human endocrine system may thus have two types of anti-inflammatory processes. Whereas both processes are associated with down-regulation of inflammatory mediator and cytokine production, they may differ in their effect on apoptosis with one (glucocorticoid) promoting apoptosis in inflammatory cells such as lymphocytes and macrophages, and the other (DHEA) protecting parenchymal cells, such as hepatocytes, from apoptosis. It has been reported that serum concentration of DHEA in humans range from 0.001 to 0.03 μM, and when given as an oral supplement the typical dose of DHEA is 25–50 mg/day [27–29]. In this study, we treated mice with DHEA at doses of 40–300 mg/kg. The doses that we used may be high relative to those used in humans, however, several reports indicate that this dose range is not excessive in rodents. Pharmacokinetics, sensitivity and other factors may be involved in the difference in dose-effects between humans and rodents. Further investigation is necessary to determine whether the normal clinical dose of DHEA is effective in treating hepatitis in human.

In conclusion, the present results suggest that DHEA can reduce T-cell-mediated inflammation in the liver. This

effect is associated with inhibition of expression of several inflammatory mediators and cytokines and inhibition of apoptosis as shown in the Con A hepatitis model. Thus, DHEA may be considered as a novel drug candidate for the therapy of liver injury. These results also suggest that the physiological role of DHEA in the human endocrine system may involve inhibition of inflammation.

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