Original Article

Effect of Dehydroepiandrosterone on Atherosclerosis in Apolipoprotein E-Deficient Mice

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***Aim*: Several clinical trials have indicated that dehydroepiandrosterone (DHEA) reduces coronary events associated with atherosclerosis. The aim of this study was to examine the inhibitory effect of DHEA on atherosclerosis and the mechanisms involved.**
***Methods*: Apolipoprotein E-knockout (apoE-KO) mice were fed an atherogenic high-cholesterol diet with or without 0.4% (w/w) DHEA for 12 weeks.**
***Results*: Although the plasma cholesterol and triglyceride levels were not decreased by DHEA, atherosclerotic lesions in the aortic sinus showed a 45% reduction in area with DHEA treatment versus untreated mice (0.19 ± 0.01 vs. 0.10 ± 0.02 μm^2; $p < 0.05$).**
Accumulation of macrophages in aortic lesions was also markedly reduced in the DHEA group, and the macrophage-positive area decreased to 0.33 ± 0.06 μm^2 from 0.67 ± 0.07 μm^2 ($p < 0.01$). Furthermore, DHEA suppressed the expression of monocyte chemoattractant protein-1 in the vessel wall. Thus, inhibition of macrophage infiltration by DHEA reduced the formation of atherosclerotic lesions in apoE-KO mice.
***Conclusions*: DHEA might be an effective agent for clinical management of atherosclerosis, but a larger controlled trial is necessary for confirmation.**

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Key words; Atherosclerosis, Dehydroepiandrosterone, Apolipoportein E-knockout mice, Monocyte chemoattractant protein-1

Introduction

Atherosclerosis is a complex vascular disease initiated by abnormal subendothelial accumulation of plasma lipoproteins in the arteries[1]. Macrophages within the vascular wall ingest cholesterol, resulting in transformation into foam cells. Concomitant activation of other cells leads to a chronic inflammatory response in the vessel walls[2].

Dehydroepiandrosterone (DHEA) and its sulfate (DHEA-S) are weak but abundant adrenal androgens, although their levels progressively decline with age in both men and women beginning in the third decade[3, 4]. A growing body of opinion suggests that DHEA supplementation may be physiologically beneficial to the elderly in a variety of ways, including prevention of cardiovascular disease[5]. Various studies concerning the links between DHEA and cardiovascular disease have produced inconsistent results, generat-

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ing much debate and controversy[6, 7]. Prospective population studies of DHEA-S and mortality in men have shown that elevated DHEA-S levels are associated with a lower mortality rate, both due to cardiovascular disease and other causes[8]. Several other longitudinal, cross-sectional, and retrospective studies have examined the relationship between DHEA-S and various aspects of cardiovascular disease. In both men and women, elevated DHEA-S levels were associated with retarded progression of atherosclerosis on coronary angiography[9]. Also, low DHEA-S levels were linked to a higher risk of CVD in some studies[10, 11], but not in others[12, 13].

Apolipoprotein E (apoE)-deficient mice show a spontaneous increase of total plasma cholesterol and triglycerides as well as decreased high-density lipoprotein. The atherosclerotic lesions developing in apoE-deficient mice have been found to display a similar morphology to those in humans[14]; therefore, we investigated the effect of DHEA on atherosclerosis in apoE-deficient mice.

Various adhesion molecules, growth factors, cytokines, and chemoattractants have been implicated in the atherosclerotic process[15]. One chemoattractant that seems to have a role in early atherogenesis is monocyte chemoattractant protein (MCP)-1, which is strongly expressed in the macrophage-rich regions of human and rabbit atherosclerotic lesions[16, 17]. Therefore, to investigate the influence of DHEA on atherogenesis *in vivo*, apoE-deficient mice were treated with DHEA, and the lesion surface area in the aortic sinus was measured and MCP-1 mRNA expression was assessed.

Materials and Methods

Mice

ApoE (−/−) mice were obtained from Jackson Animal Laboratory (Bar Harbor, ME). To create atherosclerosis, mice were placed on the following diet for the indicated period.

The animal care and procedures of the experiments were approved by the animal care committee of Yokohama City University.

Diet

In the first experiment, 8-week-old apoE-deficient male mice were given a high fat diet containing 20% (g/100 g) fat and 0.2% cholesterol for 2 weeks before the treatment period. Mice were then divided into two groups ($n = 12$ per group): one group received DHEA added to the diet (0.4% w/w corresponding to 100 mg/kg/day) and the other group (control) was maintained on the basal diet. DHEA was mixed into the pelletized diet by Oriental Co., Ltd. The dose tested was the same as in previous studies[18, 19], and the treatment period was 12 weeks. Animals were housed in a room with a 12-h light/dark cycle and were allowed free access to food and water. At the end of the study, animals were anesthetized by inhalation of ether after a 16-h fast, and blood was collected from the retro-orbital plexus for further analysis.

Histological Assessment of Atherosclerotic Lesions

Several mice from each group were killed at the age of 22 weeks (after 12 weeks on the atherogenic diet, respectively). The proximal aorta and the part of the heart containing the aortic root were removed, equilibrated in 20% sucrose, and embedded in OCT compound (Tissue-Tek). Cryosections (10 μm thick) spanning a 550-μm region of the aortic root were cut, and then 5 sections (every 10th section, i.e., 100 μm apart) from each mouse were stained with Sudan Ⅲ and hematoxylin[20]. For quantitative analysis of atherosclerotic lesions, the total lesion area in the 5 sections from each mouse was measured with NIH Imaging Software according to modification of the method described by Paigen *et al.*[21].

Measurement of Lipoproteins

If HPLC is performed by an on-line single detection technique combined with a selective enzymatic reaction, this allows the lipid constituents of lipoproteins in the column effluent to be detected and monitored without the need for subsequent analysis of column fractions. The on-line detection technique eliminated laborious and time-consuming procedures associated with fraction collection and achieved a high throughput of samples, while improving analytical precision and detection sensitivity; however, this method requires the separate injection of each enzyme reagent, which is both inefficient and wasteful. Multiple injections may be impossible for small samples, e.g., those from individual mice. Usui *et al.*[22] developed a new dual detection HPLC system for lipoprotein analysis that made it possible to simultaneously measure cholesterol and triglycerides in a single sample, thus reducing the number of analytical runs and tests needed. Therefore, plasma lipoproteins at the age of 22 weeks were analyzed by an on-line dual enzymatic method that allowed simultaneous quantification of cholesterol and triglycerides by HPLC at Skylight Biotech Inc. (Akita, Japan) according to the procedure described by Usui *et al.*[22]. In brief, 200 μL serum diluted to 1:20 with saline was injected into two TSK gel LipopropakXL columns (300 × 7.8 mm; Tosoh) connected in tandem, and the levels of cholesterol and

triglycerides in lipoproteins separated by size were determined using enzymatic reagents prepared by Kyowa Medex (Tokyo, Japan). Total cholesterol and triglyceride concentrations (mg/dL) were calculated by comparing the total area under the chromatography curves with calibration standards of known concentrations[23].

Immunohistochemistry

Serial cryosections of the aortic root harvested at 100-μm intervals were stained with an anti-mouse monocyte/macrophage monoclonal rat antibody (BM8; Hycult Biotecnology, 1:250 dilution) or an anti-human T-cell polyclonal rabbit antibody (CD3; DAKO; 1:100 dilution), followed by detection with biotinylated secondary antibodies and streptavidin-horseradish peroxidase[24]. Quantitative analysis of the stained area was performed as described previously, and the percent positive area ((stained area/total atherosclerotic lesion area) × 100) was calculated[25].

Isolation of RNA and Real-Time PCR Analysis

Aortic arch tissues at the age of 22 weeks were washed briefly with 1 × PBS before RNA extraction. They were homogenized and total RNA was extracted with Trizol reagent (Gibco-BRL Life Technologies). The quantity and quality of each RNA sample were evaluated by spectrophotometry (Beckman Coulter, DU640B) at 260 and 280 nm. RNA quality was also examined by electrophoresis on 1% formaldehyde agarose RNA gel. Monocyte chemoattractant protein-1 (MCP-1) gene expression was analyzed by real-time quantitative RT-PCR using the TaqMan system with real-time detection of fluorescence (ABI Prism 7700; Perkin-Elmer Inc.). Expression of the target gene sequence was normalized for the expression of an endogenous control, glyceraldehyde-3-phospate-dehydrogenase (GAPDH) (GAPDH TaqMan Control Reagent Kit; ABI Prism 7700, Perkin-Elmer Inc.). Primers and TaqMan probes for MCP-1 and the reference (GAPDH) were constructed using Primer Express (ABI Prism 7700; Perkin-Elmer Inc.). The following forward (F) and reverse (R) primers and TaqMan probes (P) were used for the quantification of MCP-1 and GAPDH mRNA: MCP-1 (F), 5'-GCTGCTACTCATTCACTGGCAA-3'; MCP-1 (R), 5'-CTGCTGCTGCTGATTCTCTTGT-3'; MCP-1 (P), 5'-TGATCCCAATGAGTCGGCTGGAGAA-3'; GAPDH (F), 5'-TGTTCTAGAGACAGCCGCATCTT-3'; GAPDH (R), 5'-CACCGACCTTCACCATATTGT-3'; and GAPDH (P), 5'-TTGTGCAGTGCCAGCCTCGTCTCATA-3'

Table 1. Plasma cholesterol and triglyceride levels

	Control group	DHEA group	*p* value
Total cholesterol (mg/dL)	1,119 ± 236	1,212 ± 144	0.39
Triglycerides (mg/dL)	75 ± 25	99 ± 29	0.11

Values are the mean ± SD for eight mice.

Table 2. Plasma cholesterol profile

	Control group	DHEA group	*p* value
Cholesterol (mg/dL)			
CM	4.4 ± 1.6	4.4 ± 1.0	0.99
VLDL	856 ± 242	953 ± 144	0.35
LDL	231 ± 23	228 ± 16	0.82
HDL	27 ± 1.7	26 ± 4.0	0.54

Values are the mean ± SD for eight mice. VLDL, Very low density lipoprotein; LDL, low density lipoprotein; CM, chyromicron; HDL, high density lipoprotein

Statistical Analysis

Data are presented as the mean ± SD. The unpaired Student's *t*-test was used to assess the significance of differences between 2 groups. For analysis of lesion size, comparisons between groups were performed by the Mann-Whitney *U* test. In these analyses, $p < 0.05$ was considered significant.

Results

DHEA did not Decrease Plasma Lipid Levels or Alter the Cholesterol Distribution in ApoE-Deficient Mice

Eight-week-old apoE-deficient mice were fed a Western diet for 8 weeks, starting from 2 weeks before DHEA supplementation (0.4% w/w in the diet, corresponding to 100 mg/kg/day). When the effect of DHEA on plasma lipids was examined, neither plasma cholesterol concentration nor missing words altered by DHEA compared with control mice (**Table 1**).

To assess whether DHEA altered the lipoprotein profile, the distribution of cholesterol in the lipoproteins was analyzed by high-resolution HPLC[22]. Very low density lipoprotein (VLDL) cholesterol was not decreased by DHEA treatment compared with the level in control animals (953 ± 144 vs. 856 ± 242 mg/dL). Similarly, DHEA did not decrease low density lipoprotein (LDL) cholesterol (228 ± 16 vs. 231 ± 23 mg/dL; **Table 2**). Also, there were no significant differences in chyromicron (CM) or high density lipoprotein (HDL) cholesterol between DHEA-treated and control mice.

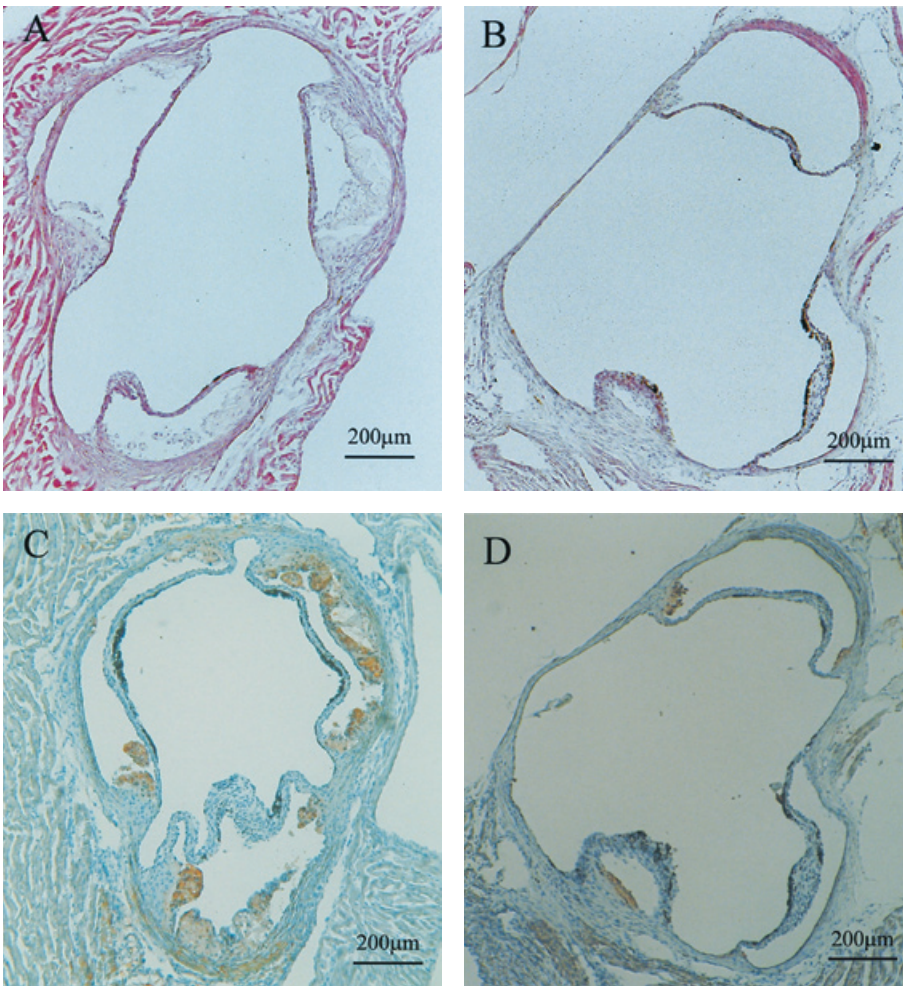

Fig. 1. Light photomicrographs of sections of aortic lesions in apo-E-KO mice maintained on the atherogenic diet for 12 weeks.

A and C, nontreated (control) group. B and D, DHEA-treated (drug) group. Sections were taken at the same level of aortic valves and stained for lipid with hematoxylin and eosin (A and B) for with Sudan Ⅲ (C and D). Representative images are shown from 8 mice at 22 weeks of age. Original magnification × 80.

DHEA Decreased the Lesion Area in the Aortic Sinus

The extent of atherosclerotic lesions was measured in serial sections obtained between the aortic valve and the aortic sinus. Representative Sudan Ⅲ-stained aortic root sections from apoE-KO mice fed an atherogenic diet are shown in **Fig. 1**. The cross-sectional lesion area was significantly smaller in the DHEA group than in the control group (45%; 0.10 ± 0.02 vs. 0.19 ± 0.01 μm^2, $p < 0.05$, **Fig. 2**).

DHEA Reduced Macrophage Infiltration into Atherosclerotic Lesions

Subendothelial macrophages were abundant in the atherosclerotic lesions of control apoE-KO mice fed an atherogenic diet for 8 weeks (**Fig. 3A**, **C**), but fewer macrophages were detected in the lesions of DHEA-treated apoE-KO mice (**Fig. 3B**, **D**). Quantitative analysis confirmed that the area of BM8-positive staining was significantly reduced in the DHEA group (0.33 ± 0.06 vs. 0.67 ± 0.07 μm^2 per five sections, $p < 0.01$; **Fig. 3E**).

Real-Time PCR

To further assess the mechanisms underlying this reduction of lesions, RNA were isolated from aortae of apo-E-KO mice ($n = 8$) with or without DHEA treatment at 22 weeks of age and MCP-1 mRNA was quantified. DHEA treatment significantly down-regulated the expression of MCP-1 mRNA (2.0 ± 0.6 vs. 1.0 ± 0.4%, $p < 0.05$; **Fig. 4**).

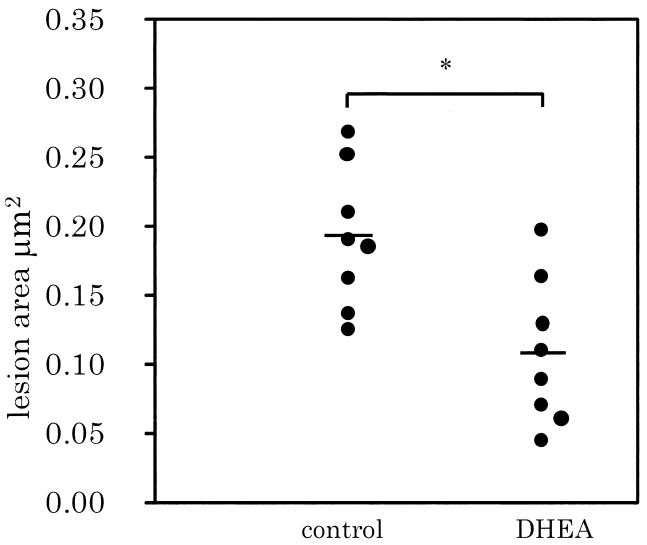

Fig. 2. Dot plot of cross-sectional lesion areas of aortic sinus in apo-E-KO mice maintained on the atherogenic diet for 12 weeks with or without DHEA-treatment.

Aortae were isolated from apo-E-KO mice ($n=8$) at 22 weeks of age. $^{*}p<0.05$

Discussion

In the present study, we demonstrated that DHEA treatment could protect apoE-knockout mice against dietary atherosclerosis. We postulated that inhibition of MCP-1 contributed to the suppression of atherosclerosis in these mice, but the possibility remains that other effects on inflammation, cell adhesion, and oxidation also enhanced the antiatherogenic activity of DHEA.

Although the plasma concentration of DHEA is usually considerably higher than that of any other androgen, little information is available concerning its direct actions on the blood vessels[26]. DHEA has been shown to have an antiatherosclerotic effect in animal models[27, 28], although the mechanism involved is not yet known. We focused on the effect of DHEA on macrophage infiltration into atherosclerotic lesions, a process that plays a central role in the initiation and progression of atherosclerosis[15]. No pharmacologic intervention that specifically targets the infiltration of monocytes and/or macrophages is clinically available at present.

DHEA has been shown to reduce the serum level of LDL-cholesterol in asymptomatic humans, although no relationship has been reported between the cholesterol level and the benefits obtained from an increase of plasma DHEA[29]. In rabbits, DHEA has been consistently found to decrease atherosclerosis. All studies[27, 28, 30, 31] performed in intact or castrated male and female rabbits treated with DHEA for 5 to 30 weeks have shown a significant reduction in the extent of spontaneous or balloon injury-induced aortic or cardiac transplant atherosclerotic lesions, independently of any changes in lipids. These findings were compatible with our results. Thus, the antiatherogenic effects of DHEA are not due to a reduction of serum lipid levels.

The mechanisms by which DHEA exerts its antiatherogenic effect remain unknown. As shown in **Fig. 2**, macrophage infiltration into atherosclerotic plaques was decreased by treatment with DHEA. In experimental studies, DHEA has been found to promote fibrinolysis[32], decrease lipid accumulation in cultured mouse foam cells[33], and limit the proliferation and migration of vascular SMC lines[34]; therefore, the antiatherosclerotic effect of DHEA might be partly mediated via the suppression of macrophage infiltration.

It is known that several cytokines have a role in atherogenesis. One chemoattractant implicated in the early stages of this process is MCP-1[16], which is strongly expressed in macrophage-rich regions of human and rabbit atherosclerotic lesions[25]. Recently, anti-MCP-1 gene therapy with a deletion mutant of the human MCP-1 gene was reported to inhibit atherosclerosis in apoE-KO mice[35]. However, few authors have addressed the question of whether DHEA suppresses MCP-1 expression. As shown in **Fig. 3**, DHEA treatment actually inhibited MCP-1 mRNA expression, an effect that could predominate over stimuli that induce the expression of MCP-1 in macrophage-derived foam cells. Quite recently, it was reported that DHEA decreases MCP-1 mRNA expression in endothelial cells[36], an observation that matches our findings; however, further studies on the validation of protein for MCP-1 expression are required.

Other mechanisms may also be involved in the antiatherosclerotic effects of DHEA, such as the conversion of DHEA to estrogen, and nitric oxide may play a role in the antiatherosclerotic effects of both DHEA and estrogen[31]. A higher serum estradiol level is associated with a lower risk of CVD events in older men[37]. In men, estrogen is mainly synthesized by local tissue aromatization of androgenic precursors released from the testis and adrenal gland, and estrogen levels do not seem to decrease with age to the same extent as those of testosterone or DHEA-S[3]. To investigate the role of estrogen, it would be interesting to examine whether an aromatase inhibitor could block the antiatherosclerotic effect of DHEA-S in apoE-KO mice and we are now planning to do such experiments.

DHEA has been shown to exert many of its

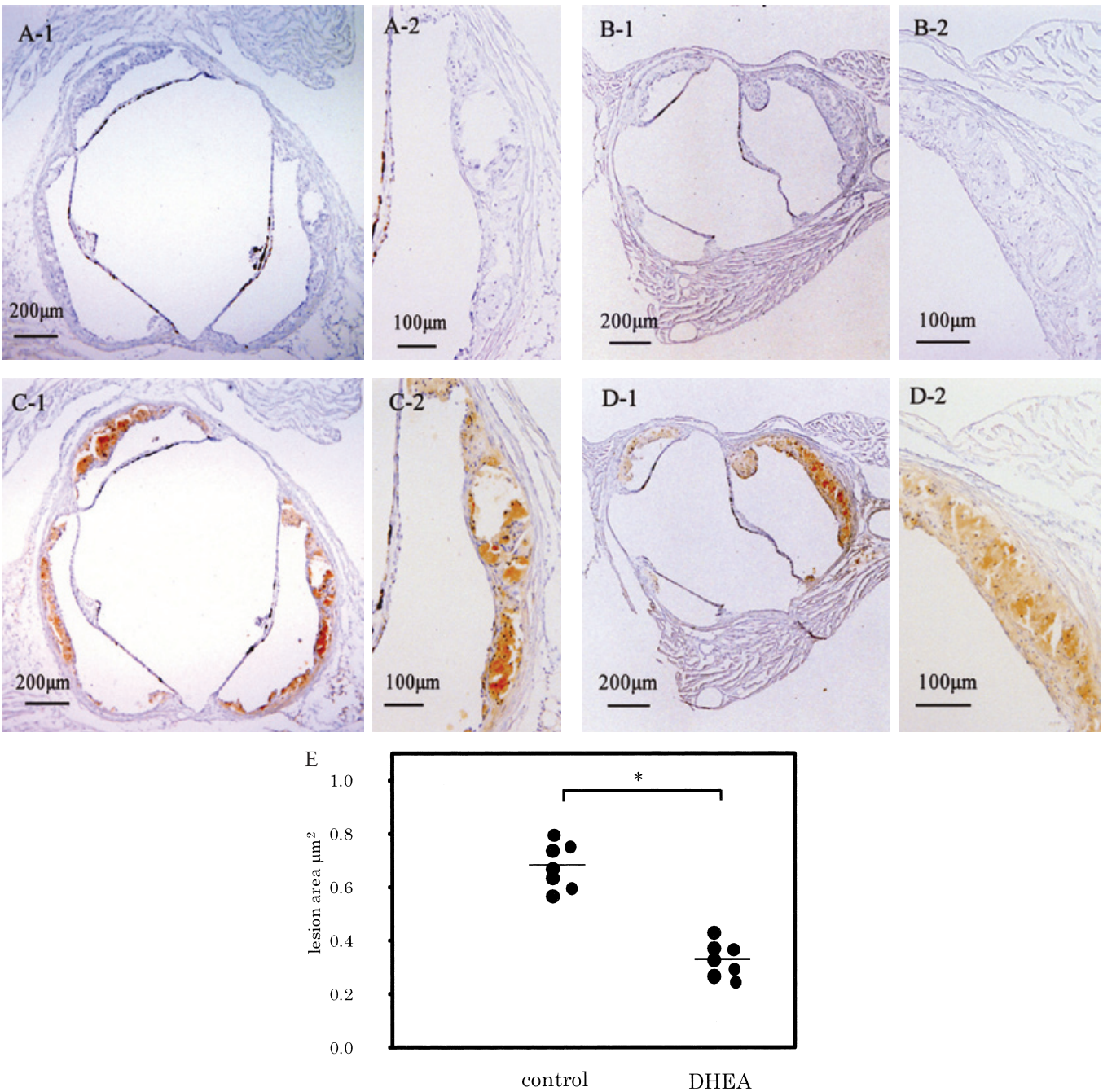

Fig. 3. Photomicrographs of immunostaining for CD3 (A and B) and BM-8 (C and D) of sections of aortic lesions in apo-E-KO mice maintained on the atherogenic diet for 12 weeks.

A and C, nontreated (control) group. B and D, DHEA-treated (drug) group. Representative images are shown from 8 mice at 22 weeks of age. Original magnification × 80 (A-1, B-1, C-1, D-1) and 160 (A-2, B-2, C-2, D-2). E, Dot plot of lesion areas with BM8-positive staining per five sections. $^{*}p < 0.05$

effects via the androgen receptor (AR) and/or estrogen receptor (ER) after enzymatic conversion to an androgen or estrogen[38]. In the elderly, the use of DHEA as a dietary supplement is of potential concern in that its androgenic or estrogenic actions may stimulate the proliferation of cancer cells within the prostate or breast and may have other adverse effects. Controversy persists as to whether DHEA enhances or reduces the

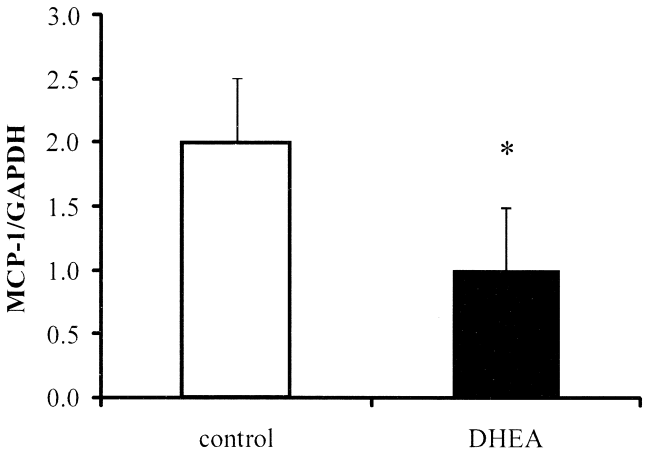

Fig. 4. Comparison of MCP-1/GAPDH mRNA expression in apo-E-KO mice maintained on the atherogenic diet for 12 weeks with or without DHEA treatment.

Aortae were isolated from apo-E-KO mice ($n=8$) at 22 weeks of age. $^{*}p<0.05$

risk of prostate and breast cancer[39]. Although DHEA and DHEAS have been reported to increase mammary cancer cell proliferation via the AR[40], various other *in vivo* and *in vitro* studies conducted with rodents and rodent cells have suggested that DHEA prevents cancer progression[41-44]. Thus, the precise influence of DHEA on normal and malignant prostate or breast cells remains to be elucidated.

Whether DHEA supplements should be given to replace declining DHEA and DHEA-S levels in aging individuals is a matter of considerable debate[6]. Claims have been made that such treatment could have beneficial effects on a variety of age-related conditions or diseases; however, discrepancies concerning the effects of DHEA in different studies have been a problem, possibly reflecting the small sample size in some instances. In this study, we found that DHEA had a protective effect against atherosclerosis in apoE-KO mice. To resolve the discrepancies regarding the benefits of DHEA, large-scale intervention trials will be necessary.

In conclusion, DHEA was shown to have a protective effect against atherosclerosis in apoE-KO mice, and inhibition of macrophage infiltration was observed after DHEA treatment.

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