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Anti-proliferative action of endogenous dehydroepiandrosterone metabolites on human cancer cell lines

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Abstract

Dehydroepiandrosterone (DHEA) is a naturally occurring steroid synthesized in the adrenal cortex, gonads, brain, and gastrointestinal tract, and it is known to have chemopreventive and anti-proliferative actions on tumors. These effects are considered to be induced by the inhibition of glucose-6-phosphate dehydrogenase (G6PD) and/or HMG-CoA reductase (HMGR) activities. The present study was undertaken to investigate whether endogenous DHEA metabolites, i.e. DHEA-sulfate, 7-oxygenated DHEA derivatives, androsterone, epiandrosterone, and etiocholanolone, have anti-proliferative effects on cancer cells and to clarify which enzyme, G6PD or HMGR, is responsible for growth inhibition. Growth of Hep G2, Caco-2, and HT-29 cells, evaluated by 3-[4,5-dimethylthiazol]-2yl-2,5-diphenyl tetrazolium bromide (MTT) and bromodeoxyuridine incorporation assays, was time- and dose-dependently inhibited by addition of all DHEA-related steroids we tested. In particular, the growth inhibition due to etiocholanolone was considerably greater than that caused by DHEA in all cell lines. The suppression of growth of the incubated steroids was not correlated with the inhibition of G6PD ($r = -0.031$, $n = 9$, NS) or HMGR ($r = 0.219$, $n = 9$, NS) activities. The addition of deoxyribonucleosides or mevalonolactone to the medium did not overcome the inhibition of growth induced by DHEA or etiocholanolone, while growth suppression by DHEA was partially prevented by the addition of ribonucleosides. These results demonstrate that endogenous DHEA metabolites also have an anti-proliferative action that is not induced by inhibiting G6PD or HMGR activity alone. These non-androgenic DHEA metabolites may serve as chemopreventive or anti-proliferative therapies.

Keywords: Dehydroepiandrosterone; Metabolites; Cancer; Anti-proliferation; Glucose-6-phosphate dehydrogenase; 3-Hydroxy-3-methylglutaryl CoA reductase

1. Introduction

Dehydroepiandrosterone (DHEA) is an endogenous steroid synthesized in the adrenal cortex, gonads, brain, and gastrointestinal tract [1,2]. Plasma concentrations of this steroid reach maximum levels during adolescence and then decline with age. Low plasma levels of DHEA have been correlated with increased risks of age-associated tumorigenesis [3,4]. DHEA prevented spontaneous tumors when given to p53-knockout mice [5] and retarded chemically induced carcinogenesis in liver [6–10], colon [11], lung [12], and breast [13,14]. In addition, DHEA inhibits proliferation of cancer [15–17] and non-cancer cells [18,19] in vitro and in vivo studies.

Although the mechanisms of the anti-proliferative action of DHEA have not yet been elucidated, there are two different explanations. First, DHEA is a potent uncompetitive inhibitor of glucose-6-phosphate dehydrogenase (G6PD) [20,21], the rate-limiting enzyme in the pentose phosphate pathway. This pathway provides ribose phosphate, which is necessary for RNA and DNA biosynthesis [8]. Indeed, many types of cancers and cultured tumor cells exhibit large increases in G6PD activity [22]. Second, DHEA may inhibit cholesterol biosynthesis and the mitogen-activated protein kinase (MAPK)-mediated signal transduction pathway for

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DNA synthesis by down-regulating 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) [23,24], the rate-limiting enzyme in cholesterol biosynthesis. Inhibition of HMGR depletes endogenous pools of mevalonic acid (MVA), the immediate product of HMGR. MVA is a precursor of farnesyl diphosphate, which is necessary for the farnesylation of Ras protein, an essential reaction in the MAPK cascade [25,26].

DHEA is often referred to as an adrenal androgen because it can be converted into testosterone and dihydrotestosterone in the peripheral tissues. Although DHEA is a naturally occurring steroid and seems to be less cytotoxic than other anti-neoplastic drugs, administration for extended periods increases circulating testosterone and dihydrotestosterone manifold above normal levels, especially in women [27], and may cause masculinization. Endogenous DHEA metabolites (Fig. 1), i.e. 7α-hydroxy-DHEA (7α-OH-DHEA), 7β-hydroxy-DHEA (7β-OH-DHEA), 7-oxo-DHEA, androsterone, epiandrosterone, and etiocholanolone, cannot be converted to testosterone. Although 7-oxygenated DHEA may be converted to 7-oxygenated testosterone, this steroid seems to have no androgenic activity [28]. Therefore, these DHEA metabolites may be more useful therapeutic agents than DHEA. However, the effects of the DHEA metabolites on cell proliferation have not been studied.

The present study was undertaken to investigate whether DHEA metabolites also have anti-proliferative effects on

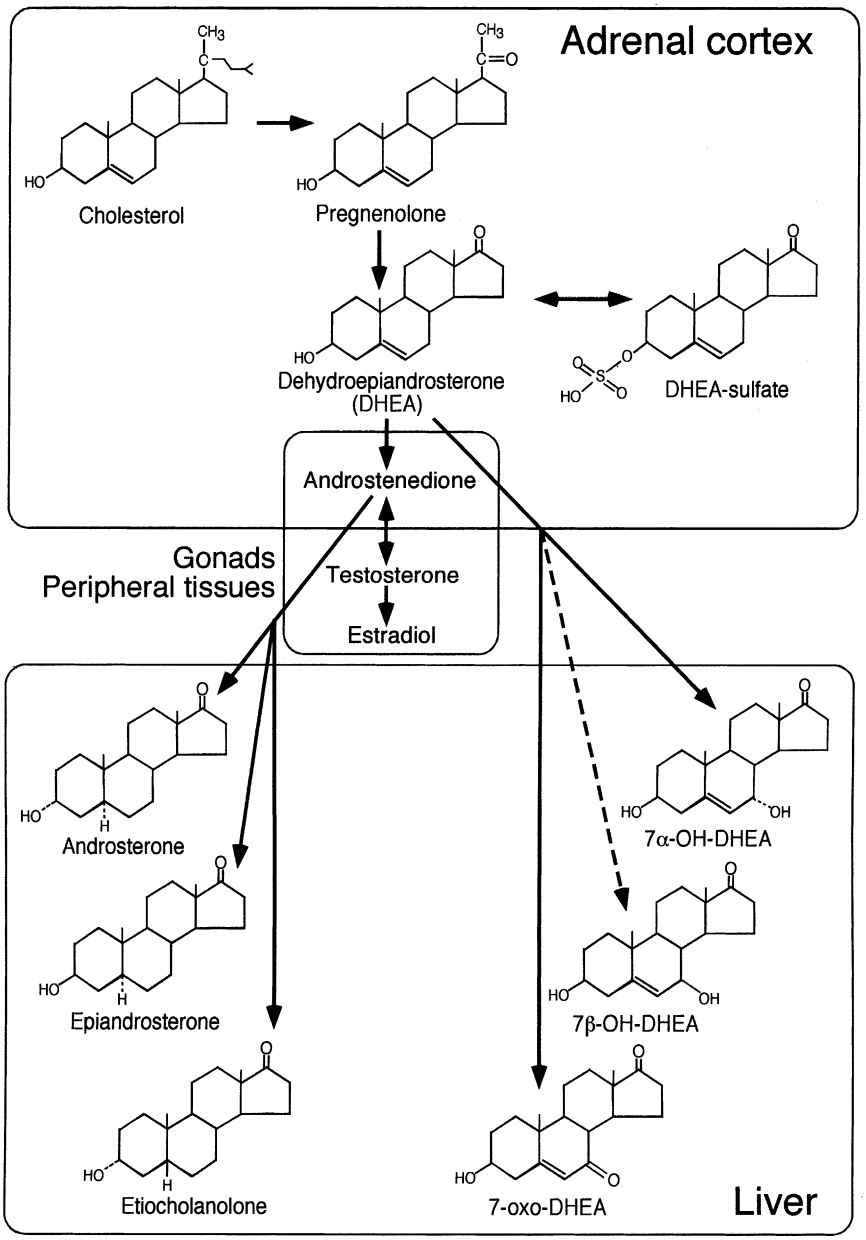

Fig. 1. Biosynthetic pathway of DHEA and related steroids from cholesterol. Solid arrows represent known enzymatic reactions whereas broken arrows represent putative reactions that have yet to be confirmed.

cancer cells and to clarify which pathway, the pentose phosphate pathway or mevalonate pathway, is responsible for growth inhibition. We used a *ras*-activated human hepatoma cell line, Hep G2 [29], and human colonic adenocarcinoma cell lines, Caco-2 [30] and HT-29 [31], and systematically studied the effects of DHEA and its metabolites on cell growth and G6PD and HMGR activities. The results showed that not only DHEA, but DHEA metabolites also had an anti-proliferative effect. However, tumor growth inhibition was not explained by the inhibition of either G6PD or HMGR activities.

2. Experimental

2.1. Chemicals

DHEA, DHEA-sulfate, pregnenolone, androsterone, epiandrosterone, and etiocholanolone were purchased from Sigma Chemical Co. (St. Louis, MO) and purified by recrystallization. RS-5-[^{3}H]mevalonolactone (MVL), (888.0 GBq/mmol) was obtained from NEN (Boston, MA). 3-Hydroxy-3-methyl-3-[^{14}C]glutaryl CoA (2.15 GBq/mmol) and γ-[^{32}P]ATP (110 TBq/mmol) were obtained from Amersham (Aylesbury, UK). All other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Syntheses of 7-oxygenated DHEA derivatives

7α-OH-DHEA, 7β-OH-DHEA, and 7-oxo-DHEA were synthesized from 3β-(*t*-butyldimethylsilyloxy)-17,17-ethylenedioxyandrost-5-en-7-one, which was obtained from DHEA in three steps (ketalization, silyl ether formation, and allylic oxidation with *tert*-butyl hydroperoxide using ruthenium chloride as a catalyst [32]). Deprotection of the enone under acidic conditions (2N HCl in methanol tetrahydrofuran) gave 7-oxo-DHEA. 7β-OH-DHEA was prepared by reduction of the enone with $NaBH_4$ in the presence of $CeCl_3$ [33] followed by removal of the protecting groups under the acidic conditions as described above. 7α-OH-DHEA was synthesized from the enone in four steps, i.e. reduction with lithium tri-sec-butylborohydride in tetrahydrofuran [33], acetylation of the resulting 7α-alcohol with Ac_2O-pyridine, removal of the silyl and ethylenedeoxy groups in the same manner as described above, and final removal of the acetyl group by 5% KOH in methanol tetrahydrofuran. The purities of these compounds were checked by gas–liquid chromatography (GLC), and each gave only a single peak.

2.3. Cell cultures

Hep G2 cells were supplied by Riken Cell Bank (Tsukuba, Japan), and Caco-2 and HT-29 cells were obtained from American Type Culture Collection (Rockville, MD). Stock cultures were grown and maintained in Eagle's minimum essential medium (Life Technologies, Inc., Grand Island, NY) supplemented with 1 mM pyruvate, 0.1 mM non-essential amino acids, and 10% fetal bovine serum (FBS). The cultures were incubated at 37 °C in a humidified incubator containing 5% CO_2, 95% air.

2.4. Growth inhibition assays

2.4.1. MTT assay

The effects of DHEA and related steroids on cell growth were tested by 3-[4,5-dimethylthiazol]-2yl-2,5-diphenyl tetrazolium bromide (MTT) assay [34]. On day 0, cells were seeded in 96-well plates at a density of 1.0×10^4 cells per well. On day 1, the original growth medium was removed and replaced with fresh medium containing various concentrations of steroids (1–100 μM) dissolved in 1% ethanol. Although 1% ethanol in the medium had no detectable effects on cell growth, the same concentration of ethanol was also added to the controls. On days 2–4, the medium was discarded, MTT solution (0.5 mg/ml) was added to all wells, and the cells were incubated at 37 °C for 4 h. Formazan products were dissolved with dimethylsulfoxide (DMSO), and the absorption at 540 nm was measured by Microplatereader M-Emax (Wako, Japan).

2.4.2. BrdU assay

Inhibition of cell proliferation by the steroids was also evaluated by bromodeoxyuridine (BrdU) incorporation assay. On day 0, 96-well plates were seeded with 1.0×10^4 cells per well. On day 1, the original growth medium was removed and replaced with fresh medium containing 100 μM of steroids dissolved in 1% ethanol. After 48 h of incubation, BrdU incorporation was measured by using the cell proliferation ELISA system, version 2 (Amersham). Briefly, BrdU labeling solution was added to each well and incubated for 2 h at 37 °C. After fixation and blocking, peroxidase-labelled anti-BrdU solution (100 μl per well) was added, and the mixture was incubated for 90 min at room temperature. At the end of the assay, tetramethylbenzidine (TMB) was added into each well as a peroxidase substrate (100 μl per well). After 5 min of incubation at room temperature, the reaction was stopped, and the absorption at 450 nm was measured by Microplatereader M-Emax. Results were expressed as a percentage of the control absorbance.

2.5. Assay of HMGR activity

HMGR activities in Hep G2 cells were measured according to the method described by Goldstein et al. [35] with minor modifications. Cells were seeded at a density of 5×10^5/12.5 cm^2 tissue culture flask. After 3 days, when the cells were about 50% confluent, the medium was replaced with fresh medium containing 50 μM of DHEA or related steroids dissolved in 1% ethanol. After 24 h of incubation, the medium from each flask was discarded, and the attached

cells were rinsed twice with phosphate-buffered saline (PBS). Cells were then harvested by use of a cell scraper and centrifugation at 10,000 × *g* for 1 min. Cell-free extracts were prepared by adding 65 μl of phosphate buffer containing 0.25% (v/v) Brij 96 to the cell pellets, and aliquots were used for determination of protein concentration [36]. The cell extract (50 μl) was incubated in a final volume of 150 μl of 100 mM potassium phosphate buffer (pH 7.4) containing a NADPH generating system. The reaction was started with the addition of 15 μl of [^{14}C]HMG-CoA and stopped after 30 min at 37 °C with the addition of 20 μl of 6N HCl. After adding unlabeled MVL (0.5 mg) as a marker for visualization on thin-layer plates, [^{3}H] MVL (50,000 dpm) as an internal recovery standard, 200 μl of ethanol, 0.2 g of anhydrous Na_2SO_4, and 50 μl of water, MVL was extracted twice with 1 ml of diethyl ether, separated by thin-layer chromatography, and measured by dual-label liquid scintillation counting. The result was expressed as a percentage of the control radioactivity measured with the cells incubated in the presence of 1% ethanol.

2.6. Assay of G6PD activity

Pooled Hep G2 cell-free extracts were used for measuring G6PD activity. Cells were seeded at a density of $1 \times 10^6/25\,cm^2$ tissue culture flask. After 4 days, when the cells were about 70% confluent, the medium from each flask was discarded, and the attached cells were rinsed twice with PBS. Cells were then harvested by use of a cell scraper and centrifugation at 10,000 × *g* for 1 min. Cell-free extracts were prepared by the same method as in the HMG-CoA reductase assay and stored at −80 °C. The reaction was started by the addition of the cell extracts (20 μl) to 980 μl of 0.1 M Tris–HCl buffer (pH 8.1) containing 1 M $MgCl_2$, 10 mM NADP, 200 mM glucose-6-phosphate, and various concentrations of steroids (1–100 μM) dissolved in 1% ethanol, and the mixture was incubated at 25 °C. The initial rate of NADPH formation was determined by measuring the increase of absorption at 340 nm [37]. The result was expressed as a percentage of the control activity measured with the cell extract incubated in the presence of 1% ethanol.

2.7. Determination of LDH activity in cells and medium

Cytotoxicity of DHEA and related steroids was assayed by measuring the activity of lactate dehydrogenase (LDH) in the cells and the medium. Hep G2 cells were seeded at a density of $5 \times 10^5/10\,cm^2$ dish. After 24 h, the medium was replaced with fresh medium containing various kinds of steroids (10–100 μM) dissolved in 1% ethanol, and the cells were incubated for 48 h. LDH activities in the medium and in the cells were measured by using a LDH CII kit (Wako). Briefly, a 10 μl aliquot of cell culture medium (released sample) or cell lysate (non-released sample) was added to 100 μl of 0.1 M Tris–HCl buffer (pH 8.4) containing 0.1 M lithium lactate, 2.6 mM NAD, 0.49 mM nitrotetrazolium blue (NO_2-TB), and 1.8 U/ml diaphorase, and the mixture was incubated at 37 °C for 10 min. LDH activity was determined by measuring the absorbance at 560 nm.

2.8. Screening for apoptosis by DNA-binding fluorochromes

Hep G2 cells were seeded at a density of $2 \times 10^6/10\,cm^2$ tissue culture dish. Medium was replaced with fresh medium containing each steroid (100 μM) dissolved in 1% ethanol. Both attached and floating cells were harvested after 72 h. The cells were fixed with 4% paraformaldehyde solution for 24 h, and DNA was stained with 0.2 mM bisbenzimide H33258 solution (Wako). The bisbenzimide H33258 solution at 1 mM in PBS was freshly prepared before each experiment. Fluorescent DNA-dye complexes were observed under a fluorescence microscope with a 340/380 nm excitation filter. About 200–300 cells of attached cells and about 30 cells of floating cells were counted at a magnification of 400× [38].

2.9. Statistics

Data are reported here as the mean ± S.D. The statistical significance of differences between the results in the different groups was evaluated with the Student's two-tailed *t*-test. We tested correlation by calculating Pearson's correlation coefficient, *r*. For all comparisons, significance was accepted at the level of $P < 0.05$.

3. Results

3.1. Effects on growth inhibition

When 10–100 μM of DHEA and related steroids were added to Hep G2 cell cultures and incubated for 24–72 h, cell growth evaluated by MTT assay was significantly inhibited in a dose- (Fig. 2) and time-dependent manner (Fig. 3). However, each steroid revealed various efficacies of inhibition. The growth inhibition rates at 100 μM after 72 h incubation in order of inhibition efficacy were: etiocholanolone (79%) > pregnenolone (70%) > epiandrosterone (67%) > DHEA (57%) > androsterone (55%) > 7α-OH-DHEA (48%) > 7-oxo-DHEA (44%) > DHEA-sulfate (35%) > 7β-OH DHEA (33%). Growth inhibition of Hep G2 cells by these steroids was also evaluated by BrdU assay, which determines DNA synthesis. The results obtained by the two different assay methods were similar, and a significant correlation was observed between the data of both assays ($r = 0.808$, $n = 9$, $P < 0.05$).

DHEA and its metabolites, except for 7β-OH-DHEA, also significantly inhibited the proliferation of other cancer cell lines, Caco-2 and HT-29 (Fig. 4). In particular, the growth inhibition due to etiocholanolone was significantly greater

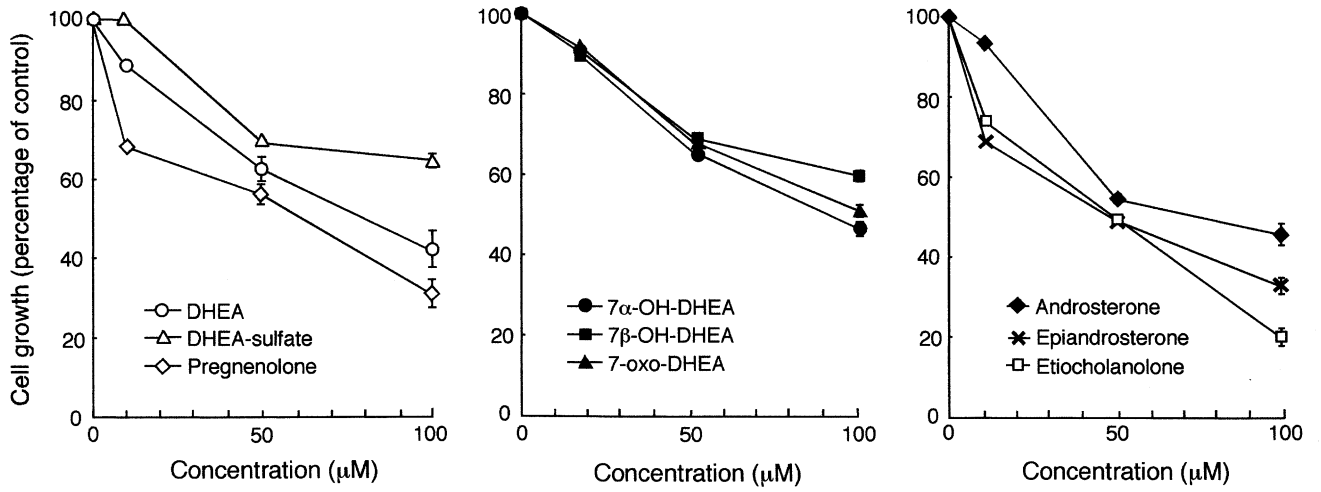

Fig. 2. Effects of steroid concentrations on cell growth. Hep G2 cells were incubated for 72 h with each steroid at concentrations of 0–100 μM. Effects of steroid concentrations on cell growth were determined by MTT assay. Each data point and error bar represent the mean ± S.D. from quadruplicate determinations. Where S.D. bars are not apparent, the S.D. was smaller than the symbol.

than that caused by DHEA at the same concentration and incubation time in all cell lines.

3.2. Evaluation of cytotoxicity

The effects of treatment with DHEA and related steroids for 48 h on cytotoxicity were studied by measuring LDH activity in medium and cells. Cell cultures exposed to each steroid at concentrations up to 50 μM showed neither a significant increase of LDH activity in the medium nor a significant decrease of the activity in cells. However, a significant increase of LDH activity in medium and a decrease of the activity in cells were observed when the cells were exposed to 100 μM of etiocholanolone (Fig. 5). To screen whether each steroid causes apoptosis to cells, we employed DNA-binding fluorochromes. After addition of each steroid at a concentration of 100 μM, a few floating cells were observed. However, no apoptotic cells were detected in both attached and floating cells.

3.3. Effects of deoxyribonucleosides (DN), ribonucleosides (RN), or MVL on DHEA or etiocholanolone-mediated growth inhibition

Hep G2 cells were treated with 50 μM of DHEA or etiocholanolone in combination with 20 μM of DN (thymidine, deoxycytidine, deoxyadenosine, and deoxyguanosine), 30 μM of RN (uridine, cytidine, adenosine, and guanosine), or 2 mM of MVL to test their ability to prevent the growth-inhibitory effects of DHEA and etiocholanolone.

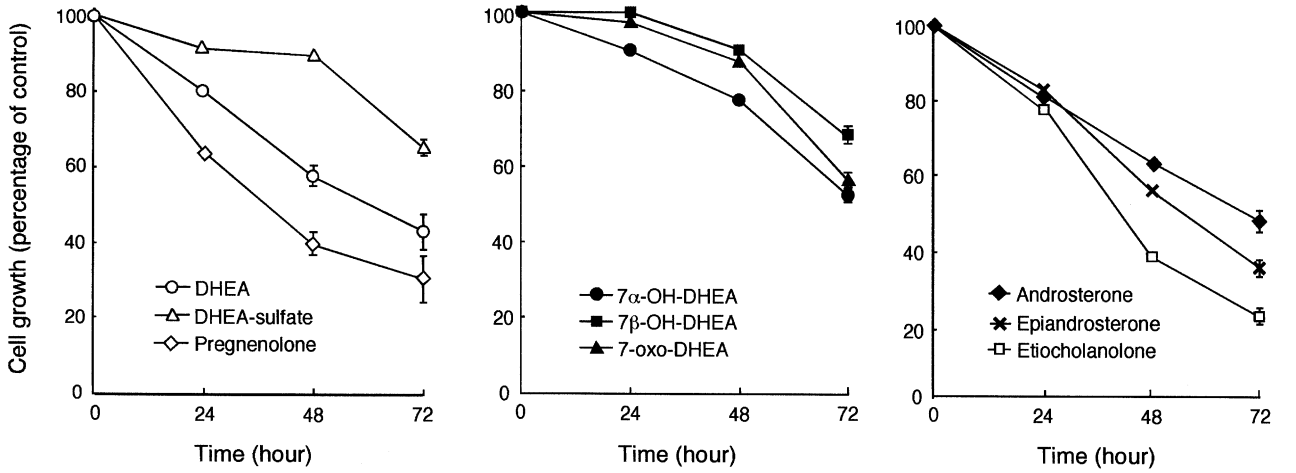

Fig. 3. Effects of incubation time on cell growth. Hep G2 cells were incubated for 0–72 h with each steroid at a concentration of 100 μM. Effects of incubation period on cell growth were determined by MTT assay. Each data point and error bar represent the mean and S.D. from quadruplicate determinations. Where S.D. bars are not apparent, the S.D. was smaller than the symbol.

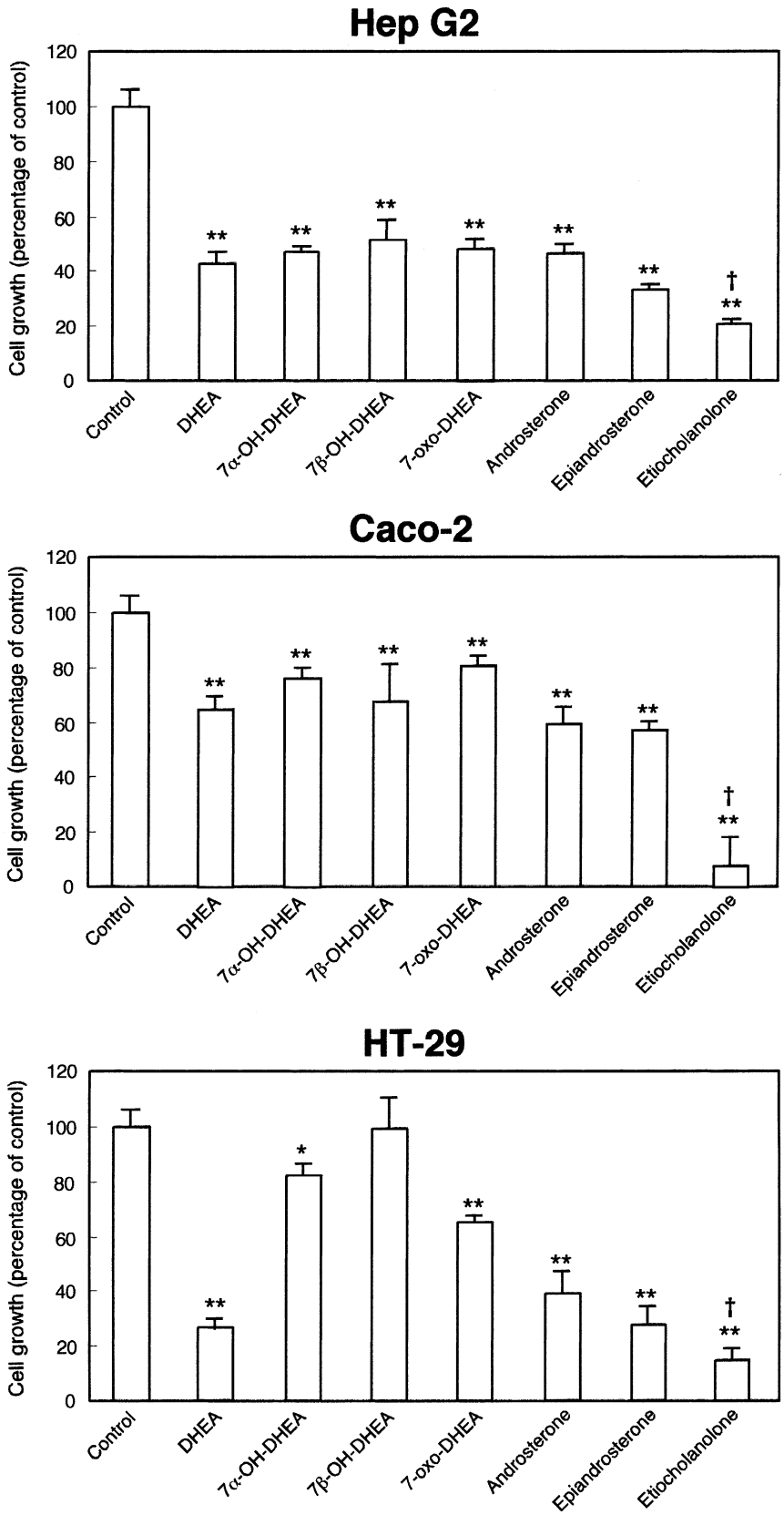

Fig. 4. Effects of DHEA and its metabolites on proliferation of different cell lines. Hep G2, Caco-2, and HT-29 cells were incubated for 72 h with each steroid at a concentration of 100 μM. Cell growth was evaluated by MTT assay. Each column and error bar represent the mean and S.D. from quadruplicate determinations. $^{*}P < 0.001$, significantly different from control; $^{**}P < 0.0001$, significantly different from control; $^{\dagger}P < 0.0001$, significantly different from DHEA.

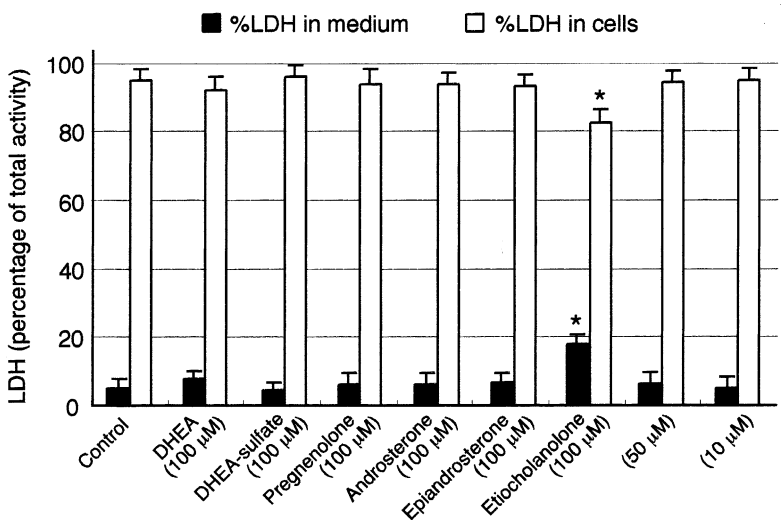

Fig. 5. Effects of DHEA and related steroids on the percentage of total activity of LDH in the Hep G2 cells and in the medium. Cells were incubated with each steroid for 48 h. Each column and error bar represent the mean and S.D. from quadruplicate determinations. $^{*}P < 0.0001$, significantly different from control.

The addition of DN or MVL to the medium did not prevent growth inhibition while the addition of RN partially overcame the growth inhibition induced by DHEA (Fig. 6A). In contrast, the growth inhibition by etiocholanolone was not prevented by the addition of DN, RN, or MVL (Fig. 6B).

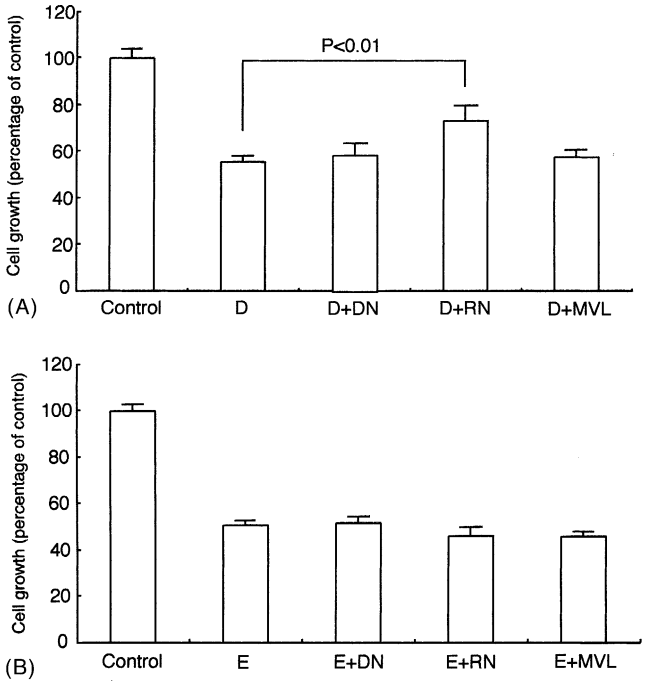

Fig. 6. Effects of DN, RN, or MVL on DHEA- or etiocholanolone-induced growth inhibition. Hep G2 cells were incubated for 48 h with 50 μM of DHEA (D), etiocholanolone (E) and DN (20 μM each), RN (30 μM each), or MVL (2 mM). Effects on cell growth were determined by MTT assay. Each column and error bar represent the mean and S.D. from quadruplicate determinations.

Further prevention of growth inhibition was not observed even when higher concentrations of DN, RN, or MVL were added to the medium.

3.4. Effects on G6PD activity

Table 1 shows the effects of adding increasing amounts of DHEA and related steroids on G6PD activity in Hep G2 cell extracts. When any steroid was added to the incubation mixture, G6PD activity was suppressed in a dose-dependent, variable manner. Epiandrosterone and DHEA were the most potent inhibitors of G6PD, while DHEA-sulfate and etiocholanolone had little effect. The inhibition rates of a 50 μM concentration of the steroids and the relative order of inhibition efficacy were: epiandrosterone (86%) > DHEA (85%) > 7α-OH-DHEA (76%) > 7β-OH-DHEA (57%) >

Table 1
Effects of each steroid on G6PD activity in Hep G2 cells

Steroid added to incubation mixture[a]	G6PD activity[b] (% of control[c])		
	1 μM	10 μM	100 μM
DHEA	82 ± 9	38 ± 3	7.4 ± 1.0
DHEA-sulfate	98 ± 3	100 ± 5	92 ± 8
Pregnenolone	75 ± 6	61 ± 2	51 ± 5
7α-OH-DHEA	92 ± 4	53 ± 4	14 ± 4
7β-OH-DHEA	97 ± 4	80 ± 1	31 ± 1
7-Oxo-DHEA	91 ± 13	78 ± 4	62 ± 6
Androsterone	99 ± 1	80 ± 1	55 ± 7
Epiandrosterone	82 ± 12	27 ± 6	9.6 ± 3.2
Etiocholanolone	104 ± 7	99 ± 7	98 ± 9

[a] Pooled Hep G2 cell-free extracts were incubated with each steroid at concentrations of 1, 10 and 100 μM, and G6PD activities were measured.
[b] Data represent the mean ± S.D. ($n = 4$).
[c] The absolute G6PD activity in the absence of steroid was 178 ± 14 nmol/min/mg protein ($n = 4$).

Table 2
Effects of each steroid on HMGR activity in Hep G2 cells

Steroid added to medium[a]	HMGR activity (pmol/min/mg protein)	
Control	137 ± 16[b]	100%[d]
DHEA	52 (42, 63)[c]	38%
DHEA-sulfate	121 (104, 138)	88%
Pregnenolone	59 (63, 56)	43%
7α-OH-DHEA	64 (66, 63)	47%
7β-OH-DHEA	58 (55, 62)	42%
7-Oxo-DHEA	133 (126, 140)	97%
Androsterone	44 (51, 37)	32%
Epiandrosterone	71 (69, 75)	52%
Etiocholanolone	99 (93, 105)	72%

[a] Hep G2 cells were exposed for 24 h to 50 μM of each steroid. The cells were then harvested for determination of HMGR activity.
[b] Mean ± S.D. ($n = 5$).
[c] Average of two assays with individual values in parentheses.
[d] Relative enzyme activities.

pregnenolone (47%) > androsterone (39%) > 7-oxo-DHEA (34%) > DHEA-sulfate (6%) > etiocholanolone (1%).

3.5. Effects on HMGR activity

The effects of 50 μM steroid treatments for 24 h on HMGR activity in Hep G2 cells are summarized in Table 2. HMGR activity was obviously inhibited by DHEA and related steroids except for 7-oxo-DHEA and DHEA-sulfate. The inhibition rates and the order of inhibition efficacy were: androsterone (68%) > DHEA (62%) > 7β-OH-DHEA (58%) > pregnenolone (57%) > 7α-OH-DHEA (53%) > epiandrosterone (48%) > etiocholanolone (28%). To evaluate whether the inhibition of HMGR activity depends on a direct interaction of the steroids with the enzyme, cell-free extracts were also incubated in the presence of 50 μM of each steroid. However, no significant inhibition of HMGR activities occurred.

3.6. Relationship between growth inhibition and G6PD or HMGR activities

Fig. 7A shows the relationship between growth inhibition evaluated by MTT assay and inhibition of G6PD activity. There was no significant correlation between inhibition of cell growth and inhibition of G6PD activities by DHEA and related steroids ($r = -0.031$, $n = 9$, NS). The relationship between inhibition of cell growth and inhibition of HMGR activity is shown in Fig. 7B, and no significant correlation was observed ($r = 0.219$, $n = 9$, NS).

4. Discussion

It has been reported that not only DHEA but also certain endogenous DHEA metabolites inhibit the activities of G6PD [20,21] and HMGR [23]. However, the effects of

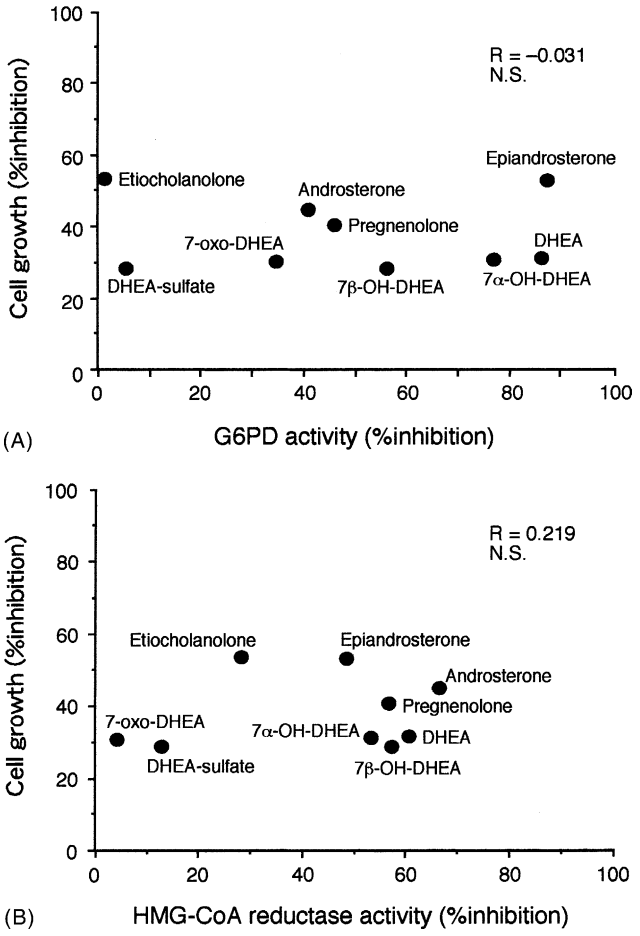

Fig. 7. Relationship between growth inhibition and inhibition of G6PD (A) and HMGR (B) activities in Hep G2 cells treated with DHEA and related steroids. G6PD activities were measured by incubating cell-free extracts with each steroid at concentrations of 50 μM. HMGR activities were determined by use of the cells exposed to 50 μM of each steroid for 24 h. Growth inhibition was determined by MTT assay (50 μM of each steroid, 72-h incubation).

these steroids on cell growth have not been tested. There are clinical reports that plasma 7α-OH-DHEA concentration [39] and urinary etiocholanolone and androsterone excretion [40,41] in breast cancer patients were lower than the concentrations in control women. In the present study, we demonstrated that many endogenous DHEA metabolites, including 7α-OH-DHEA, etiocholanolone, and androsterone, had anti-proliferative effects. In addition, we investigated whether the pentose phosphate pathway (G6PD) or the mevalonate pathway (HMGR) is more responsible for the growth inhibition.

It has been reported that ketonization of the C-17 or C-20 position and 3β-hydroxylation are required for the inhibition of G6PD activity [42]. The mechanism is uncompetitive with respect to both NADP and glucose-6-phosphate and is probably due to the binding of the steroids to the ternary enzyme–coenzyme–substrate complex(es) [21]. Since the inhibition was not caused by decreased expression of G6PD protein [19], we directly added each steroid to the G6PD

assay mixture and measured enzyme activity in vitro. If the anti-proliferative effect of DHEA-related steroids depends on the inhibition of G6PD activity, there ought to be a significant relationship between growth suppression and the inhibition of G6PD activity. However, the activity was not correlated with growth inhibition at all (Fig. 7A), suggesting that the anti-proliferative effect of these steroids is not explained by the inhibition of G6PD alone. A recent report demonstrated that DHEA had growth inhibitory effects in G6PD deficient cells (89 cells) [43], which lends support to our results that the inhibition of G6PD is but one of the anti-proliferative mechanisms of DHEA-related steroids.

HMGR is the rate-limiting enzyme in the biosynthesis of farnesyl diphosphate, which is a precursor of cholesterol and is also necessary for the farnesylation of Ras protein. In the MAPK mediated cell proliferation signaling pathway, activated Ras (farnesylated and bound GTP) switches on Raf1 kinase [44], which phosphorylates MEK1 (MAP kinase kinase) [45], and in turn, phosphorylates $p44^{MAPK}$ (ERK1) and $p42^{MAPK}$ (ERK2) [46,47]. These MAPKs can phosphorylate a variety of substrates, including Fos and Jun [48]. Fos and Jun are protein products of c-fos and c-jun proto-oncogenes, respectively, and are components of the transcription factor, activator protein-1 (AP-1) [49]. Binding of AP-1 to the DNA regulatory sites activates transcription of a variety of target genes that leads to initiation of DNA synthesis and eventually, to mitosis [50]. A recent study using cultured airway smooth muscle cells showed that DHEA decreased DNA binding of AP-1 [51]. Although the exact mechanism has not been elucidated, one possibility is that it may be due to inhibition of a kinase, which mediates AP-1 phosphorylation events that are necessary for DNA binding [50,52,53]. In our studies, however, HMGR activity was not correlated with growth inhibition (Fig. 7B), and the addition of MVL to the medium did not mitigate the growth inhibition induced by DHEA (Fig. 6). Therefore, the anti-proliferative action of DHEA is not explained by the inhibition of cholesterol biosynthesis or the MAPK signal transduction pathway due to suppression of HMGR activity. Direct interference with the MAPK pathway may be another possible mechanism for the growth inhibition by DHEA.

In the liver, DHEA is metabolized to multiple products, including 7-oxygenated derivatives [54], androsterone, epiandrosterone, and etiocholanolone (Fig. 1). Although many unique biological effects of DHEA have been reported, some may be due to metabolism of DHEA to more biologically active products. In the present study, all DHEA metabolites we tested showed anti-proliferative actions. In particular, the growth inhibition due to etiocholanolone was significantly greater than that caused by DHEA (Fig. 4). However, it is possible that the inhibitory action due to etiocholanolone and DHEA are caused by different mechanisms. DHEA markedly inhibited G6PD activity, and the anti-proliferative effects of DHEA were diminished by the addition of RN. In contrast, etiocholanolone did not inhibit G6PD activity, and the growth inhibition by etiocholanolone was not affected by the addition of RN. It may be mentioned here that G6PD is the rate-limiting enzyme in the pentose phosphate pathway, which provides ribose-5-phosphate. A decrease in the liver content of ribulose-5-phosphate, the immediate precursor of ribose-5-phosphate, was reported in rats treated with DHEA [55]. DN is synthesized from RN while RN is not synthesized from DN, but from ribose-5-phosphate, which may be the reason why the addition of DN did not diminish the inhibitory action of DHEA (Fig. 6).

A previous report showed that DHEA had a cytostatic effect and arrested cells in the G_1 phase of the cell cycle [15]. In fact, except for etiocholanolone, the steroids we tested did not cause cytotoxicity or apoptosis at a concentration of 100 μM. However, 100 μM of etiocholanolone significantly increased LDH activity in the medium and decreased the LDH activity in cells (Fig. 5), which suggests that, in contrast to DHEA and other DHEA metabolites, etiocholanolone has a cytotoxic effect at a concentration of 100 μM. It should be noted that to determine HMGR activity and to test the ability of DN, RN, and MVL to prevent growth inhibition by the steroids, we exposed cells to 50 μM of each steroid. This concentration of etiocholanolone did not change LDH activity in neither medium nor cells, which shows that 50 μM of etiocholanolone was not cytotoxic.

In summary, this study demonstrated that endogenous DHEA metabolites, i.e. DHEA-sulfate, 7-oxygenated DHEA derivatives, androsterone, epiandrosterone, and etiocholanolone, produced anti-proliferative effects on cancer cell lines. Since the growth inhibitory effects were not correlated with the inhibition of G6PD nor HMGR activity, some other mechanisms of the growth inhibition may exist. However, we can not exclude the possibility that the anti-proliferative effects of any one of the individual steroids may have been mediated via the inhibition of G6PD and/or HMGR. These non-androgenic DHEA metabolites may serve as chemopreventive or anti-proliferative therapies.

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