Dehydroepiandrosterone Can Inhibit the Proliferation of Myeloma Cells and the Interleukin-6 Production of Bone Marrow Mononuclear Cells from Patients with Myeloma

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Abstract

The serum levels of an adrenal sex hormone, dehydroepiandrosterone (DHEA), are significantly more decreased in human myelomas compared with the reduction brought by physiologic decline with age. In order to clarify the effect of DHEA on myeloma cells, we investigated whether DHEA and DHEA-S could inhibit interleukin-6 (IL-6) production of bone marrow mononuclear cells and the proliferation of myeloma cells from patients with myeloma. DHEA-S and DHEA suppressed IL-6 production from a bone marrow stromal cell line, KM-102, as well as in bone marrow mononuclear cells from patients with myeloma. Furthermore, DHEA inhibited in vitro growth of the U-266 cell line and primary myeloma cells from the patients, as well as the in vivo growth of U-266 cells implanted i.p. in severe combined immunodeficiency-hIL6 transgenic mice. DHEA up-regulated the expression of peroxisome proliferator–activated receptor (PPAR), PPARβ, but not PPARγ or PPARα, and the expression of IκBα gene in myeloma cells and bone marrow stromal cells, which could explain the suppressive effect of DHEA on IL-6 production through the down-regulation of NF-κB activity. Therefore, these data revealed that DHEA-S, as well as DHEA, had a direct effect on myeloma and bone marrow stromal cells to inhibit their proliferation and IL-6 production, respectively. (Cancer Res 2005; 65(6): 2269-76)

Introduction

Multiple myeloma is a human hematopoietic malignancy characterized by the clonal expansion of malignant plasma cells in the bone marrow. Interleukin-6 (IL-6) is a major growth factor for human myeloma cells (1–3), and an increase in serum IL-6 could be responsible for the expansion of myeloma cells and the progression of myelomas (4–8). Recently, it was further clarified that myeloma cells were heterogeneous with respect to phenotype as well as morphology, and the proliferating subpopulations of myeloma cells, MPC-1-negative (MPC-1−/C0−) immature myeloma cells, responded directly to IL-6 to proliferate, but were limited and included in the cells expressing CD45 antigen (MPC-1−/CD45+ myeloma cells; refs. 9–11).

On the other hand, the serum levels of IL-6 are considered to increase with age (12), and the age-related increase in serum IL-6 could be related to age-dependent diseases such as rheumatoid arthritis (13), atherosclerosis (14), Alzheimer’s disease (15), and B cell malignancies (16) including myelomas. The onset of these disorders in elderly people is a common feature, which has stimulated intensive research in age-related causative factors. Although the physiologic factors affecting the level of serum IL-6 with age remain to be fully clarified, here we focus on the age-associated decline in the serum dehydroepiandrosterone sulfate (DHEA-S; ref. 17). DHEA and the DHEA-S are adrenal sex hormones, and are produced the most among other adrenal cortical hormones in the human adrenal cortex (18). DHEA-S is the hormone pool of DHEA and is a good serum marker for DHEA availability. Recent reports also show that the age-associated decline in serum DHEA-S is closely related to the increase in serum IL-6 (19), and DHEA has anti-inflammatory properties such as suppression of inflammatory cytokine production. In this paper, we investigated whether serum DHEA-S levels were decreased significantly in overt myelomas compared with those in control subjects such as healthy subjects, patients with malignant lymphomas, or macroglobulinemia, and whether DHEA had a direct effect on the proliferation of myeloma cells and the IL-6 production of bone marrow mononuclear cells (BMMNC) from patients with myeloma.

Materials and Methods

Myeloma Patients. Overt myelomas and monoclonal gammapathy of undetermined significance (MGUS) were diagnosed by the criteria of Southwestern Oncology Group. In this study, 180 cases of overt myeloma and 60 cases of MGUS were registered prior to treatment. Also, healthy donors (43 cases) and patients with other hematologic malignancies such as malignant lymphoma (39 cases) and macroglobulinemia (10 cases) were registered. Bone marrow aspiration was done after receiving informed consent.

DHEA-S Concentration in the Serum and Bone Marrow. In the serum and plasma from bone marrow aspirates, DHEA-S concentrations (ng/mL) were measured by RIA method (DPC DHEA-S kit; Diagnostic Products Corporation, Los Angeles, CA). DHEA and DHEA-S were purchased from Sigma Chemicals (St. Louis, MO).

IL-6 Concentration. Human IL-6 concentrations (pg/mL) in the serum and plasma from bone marrow aspirates were measured by the high-sensitivity colorimetric sandwich ELISA method (HS600; R&D Systems, Minneapolis, MN).

Flow Cytometric Analysis. BMMNC were isolated from bone marrow aspirates from patients with MGUS or overt myelomas, and were stained with FITC-anti-CD38 antibody, PE-anti-CD19, and PC-5-anti-CD56 antibody (Coulter, Hialeah, FL), or PE-anti-MPC-1 antibody (Japan Immunoresearch Laboratories, Co., Ltd., Takasaki, Japan; ref. 20), or PC-5-anti-CD45 antibody (Coulter). The stained cells were analyzed by a flow cytometer (Epics Elite ESP, Coulter; ref. 21).
In vitro Growth of Myeloma Cell Lines and BMMNCs from Patients. Myeloma cell lines or BMMNC from the patients were cultured with DMSO control, DHEA, DHEA-S, or peroxisome proliferator–activated receptor (PPAR) agonists [WY14643, Carbacyclin, or Troglitazone (Sigma)] in RPMI 1640 + 10% FCS for 2 or 7 days. DHEA, DHEA-S, WY14643, Carbacyclin, or Troglitazone were dissolved in DMSO.

In vivo Growth of Myeloma Cell Lines in SCID-hIL6 Mice. Human myeloma cell lines (U-266, 1 × 10⁶ cells), were injected i.p. with 0.6% agarose gel into severe combined immunodeficiency (SCID)-hIL6 transgenic mice which were provided from the Chugai Institute (Tokyo, Japan; ref. 22). Eight or twelve weeks after injection, the mice were sacrificed and the mass formed in the peritoneal cavity and peritoneal lavage were harvested. The cell suspensions from samples were stained with PE-anti-human CD54 antibody (Coulter) to detect human plasma cells, and were analyzed by a flow cytometer. Animal experiments were done in accordance with institutional guidelines approved by the

![Diagram](image_url)

**Figure 1.** Decreased serum DHEA-S and increased IL-6 in overt myelomas and some cases of MGUS. A, serum DHEA-S concentrations (ng/mL) were measured by the RIA method. In males, 20 healthy donors (for age 60, the median values were 1,233.3 ng/mL; △), 14 MGUS cases without significantly decreased serum DHEA-S (for age 60, the median values were 566.7 ng/mL; ○), 30 overt multiple myeloma (for age 60, the median values were 466.7 ng/mL; *), 20 malignant lymphoma (for age 60, the median values were 1,200.8 ng/mL; △), and 5 cases with macroglobulinemia (for age 60, the median values were 1,100.0 ng/mL; ▲) were analyzed. In females, 11 healthy donors (for age 60, the median values were 2.2 pg/mL; ●), 7 MGUS cases without the significantly decreased serum DHEA-S (for age 60, the median values were 187.5 ng/mL; △), and 6 MGUS cases with the significantly decreased serum DHEA-S (for age 60, the median values were 133.3 ng/mL; □) were also analyzed. C, IL-6 concentrations (pg/mL) in the plasma from bone marrow aspirates were measured by highly sensitive ELISA. In males, 8 healthy donors (for age 60, the median values were 1.8 pg/mL; ○), 7 MGUS cases without the significantly decreased serum DHEA-S (for age 60, the median values were 2.4 pg/mL; △), 9 MGUS cases with the significantly decreased serum DHEA-S (for age 60, the median values were 16.5 pg/mL; ●), and 21 overt multiple myeloma (for age 60, the median values were 38.8 pg/mL; △), 6 MGUS cases without the significantly decreased serum DHEA-S (for age 60, the median values were 3.0 pg/mL; △), 11 MGUS cases with the significantly decreased serum DHEA-S (for age 60, the median values were 18.0 pg/mL; ●), and 14 overt multiple myeloma (for age 60, the median values were 36.0 pg/mL; *).
Animal Care Committee of Yamaguchi School of Medicine (approved no. 14-004).

Reverse Transcription-PCR for PPAR, IL-6 and InB Gene Expression. Total RNA was extracted as previously described (9). Sequences of the primers used for the PPARβ gene (23): sense 5'-CCAGATTTTAG-GACGCTGTC-3', antisense 5'-AAGTTTCTCAATGAGCCAGC-3'; for the PPARα gene: sense 5'-AATGCGAGATGGCCTA-3', antisense: 5'-GTCTCATGTGCTGATGAC-3'; for the PPARγ gene: sense 5'-TCTC-TCCGTATGGAAGACC-3', antisense 5'-GAATATAGACATCCCCAC-3'; for the IL-6 gene: sense 5'-ATGAATCTTCCCTTCCACAGGCGG-3', antisense 5'-GAAGAGGCCTCAAGGTGACT-3'; for the Il-10 gene: sense 5'-CCATGTTCAGGGCAGG-3'; antisense 5'-TGAAGAGGGCCAGTGCTC-3'.

Western Blot Analysis. Cell lysates were prepared by adding lysis buffers followed by Western blot analysis as described elsewhere (24). Specific antibodies for Il-10 and Xiap were purchased from Cell Signaling Technology (Beverly, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

Flow Cytometric Analysis of NF-κB Translocation in Nuclei Preparations. Nucleic preparations were obtained by incubating the cells with 200 μL of PIPES-Triton buffers (10 mmol PIPES, 0.1 mol/L NaCl, 2 mmol MgCl2, and 0.1% Triton X-100) for 30 minutes at 4°C. The nuclei were incubated with PE-labeled anti-NF-κB (p65) antibody (sc-8008; Santa Cruz), or PE-labeled control mouse immunoglobulin G1 (IM-0670, Coulter) for 30 minutes at 4°C and analyzed by a flow cytometer.

Statistical Analysis. Statistical analysis was conducted using Student’s t test, nonparametric Mann-Whitney U test or analysis of covariance by using Statistical Package for the Social Sciences software (SPSS Japan, Inc., Tokyo, Japan). Statistical significance was defined as P < 0.05.

Results

Decreased DHEA-S Levels in Overt Myelomas and Some Cases of MGUS. Serum levels of DHEA-S physiologically decline with age, and are higher in males than in females. We examined 27 cases of male MGUS and 23 cases of female MGUS as well as 30 cases of male and 25 cases of female overt myelomas prior to treatment. Data was analyzed separately for males and females. In males, serum DHEA-S levels were significantly decreased in 30 overt myeloma cases and 14 cases of MGUS (P < 0.01), while 20 cases of malignant lymphoma, 5 cases of macroglialnmenia, and 13 cases of MGUS showed no significant suppression of serum DHEA-S levels comparable to those in the 20 healthy donors (Fig. 1A). The same pattern of findings was also seen in female subjects (Fig. 1A). Therefore, overt myeloma cases prior to treatment showed decreased serum DHEA-S levels and some MGUS cases also showed decreased serum DHEA-S, but other MGUS cases did not. In order to confirm the decrease in DHEA-S levels in the bone marrow, we examined DHEA-S levels in the bone marrow aspirates from the 35 cases of overt myeloma (21 males and 14 females). DHEA-S levels in the bone marrow aspirates showed a significant decrease in overt myelomas in males and in females (P < 0.01) compared with those in healthy donors (Fig. 1B).

Furthermore, because the serum levels of DHEA-S are inversly correlated with the concentration of IL-6, we examined the IL-6 concentration in the plasma of bone marrow aspirates of patients with overt myeloma and MGUS prior to treatment. IL-6 concentrations in the bone marrow of overt multiple myeloma were more significantly increased than those in healthy donors, IL-6 concentrations were also increased in MGUS cases with significantly decreased serum DHEA-S, but not in MGUS cases without significantly decreased serum DHEA-S as shown in Fig. 1C.

DHEA Inhibited Production of IL-6 from a Bone Marrow Stromal Cell Line and BMMNC from Patients with MGUS and Myeloma. In order to clarify the relationship between decreased DHEA-S concentration and increased IL-6 activity in the serum and bone marrow, we examined the effect of DHEA on IL-6 production from a bone marrow stromal cell line, KM-102, BMMNC from 12 patients with overt myeloma before treatment, and 8 cases of MGUS. DHEA significantly inhibited IL-6 production from KM-102 cells as well as an IL-6-producing myeloma cell line, U-266 (data not shown; P < 0.05; Fig. 2). In primary BMMNC from two cases of MGUS and three cases of overt multiple myeloma, IL-6 concentrations in these culture supernatants were significantly inhibited by the addition of DHEA (1 × 10^-6 mol/L).

DHEA-S Inhibited In vitro Growth of Human Myeloma Cell Lines and Primary Myeloma Cells from Patients with Myeloma. Both DHEA and DHEA-S could inhibit proliferation of several kinds of human myeloma cell lines: U-266, an IL-6 responsive but independent and IL-6-producing cell line; NOP-2, an IL-6 independent cell line; and IL-KM3, an IL-6-dependent cell line (Fig. 3A).

BMMNC from bone marrow aspirates of the 34 cases of overt myeloma prior to treatment were cultured with DMSO, DHEA, or DHEA-S (1 × 10^-6 mol/L). After 7 days, the cells were harvested and IL-6 concentration was measured by ELISA. Triplicate values were statistically analyzed by Student’s t test. **, P < 0.01; *, P < 0.05.
and stained with FITC-CD38, PE-MPC-1, and PC5-CD45 antibody to analyze the subpopulations of myeloma cells that survived in the culture. Representative data (MM1, MM2, MM3, and MM4) of the 34 overt myelomas are shown in Fig. 3B. In MM1 and MM2, both MPC-1⁻ immature myeloma cells and MPC-1⁺ mature myeloma cells were significantly decreased by the addition of DHEA, compared with those in DMSO control (P < 0.01 or P < 0.05). On the other hand, in MM3, MPC-1⁻ immature myeloma cells, but not MPC-1⁺ mature myeloma cells, were decreased, whereas neither MPC-1⁻ nor MPC-1⁺ cells were decreased in MM4.

**DHEA Inhibited In vivo Growth of the Human Myeloma Cell Line, U-266 in SCID-hIL6 Transgenic Mice.** The U-266 cell line can be transplanted i.p. with agarose gel, and about 10 weeks after injection, mass formation in the agarose gel is detectable in the peritoneal cavity of SCID-hIL6 transgenic mice (22). Simultaneous and consecutive injection of DHEA (100 g/mouse) three times every week resulted in almost complete failure of i.p. implantation and mass formation of U-266 cells (Fig. 4A and B). Harvesting of peritoneal lavage showed that almost no U-266 cells were detected by staining the cells with anti-human CD54 antibody in SCID-hIL6 transgenic mice treated with DHEA. At 12 weeks after peritoneal injection of U-266 cells with s.c. injections of DHEA, all mice were alive with less mass formation (mass formation found in one out of five mice, zero out of five mice, and two out of five mice in three experiments), although without DHEA treatment, most mice showed mass formation (Fig. 4B). Thus, DHEA injection induced dramatic inhibition of i.p. transplantation of U-266 myeloma cell lines.

**DHEA Increased Expression of the PPARβ Gene and Suppressed Expression of the NF-κB Target Genes, such as IL-6, IkBα, and Xiap in U-266 and KM-102 Cells.** DHEA suppressed IL-6 production from the myeloma cell line U-266, the human bone marrow stromal cell line KM-102, and primary BMMNC from patients with MGUS and myeloma prior to treatment. First, we confirmed that both DHEA and DHEA-S induced...
augmentation of PPAR gene expression; DHEA-S enhanced expression of PPARβ but not PPARγ gene in U-266 and KM-102 cells (Fig. 5A), and PPARδ gene was not detected in either U-266 or KM-102 cells (data not shown). Because IL-6 gene expression is down-regulated by the decreased activation of NF-κB, we examined whether DHEA-S induced increased expression of the IkBα gene at an early phase (within 3 hours) in KM-102 or U-266 cells. U-266 cells were injected as described in A, and after 12 weeks, mice were sacrificed and mass formations in the peritoneal cavity were observed.

Proliferation of U-266 Cells Inhibited by the Addition of PPAR Agonists. U-266, a myeloma cell line, was cultured with the PPAR agonists (WY14643, Carbacyclin, or Troglitazone) at various concentrations for 2 days, and viable cell number was examined by the flow cytometer. As shown in Fig. 6, viable cell number of U-266 cells was decreased significantly in the presence of WY14643, Carbacyclin, or Troglitazone at concentrations of 10, 50, or 100 μmol/L. Therefore, this data shows that PPAR agonists can directly inhibit the proliferation of myeloma cell lines.

Figure 4. DHEA inhibited in vivo growth of U-266 cell lines. A, treatment with DHEA markedly inhibited recovery of U-266 cells in peritoneal lavage. U-266 cells (1 × 10⁶ cells) were i.p. injected with agarose gel in SCID-hIL6 transgenic mice, and with or without s.c. injection of DHEA (100 μg per mouse, every 3 weeks). After 12 weeks, peritoneal lavage was harvested for detection of U-266 cells by flow cytometry. Points, mean; bars, ± SD number of harvested cells (CD54+) per milliliter in triplicate. **, P < 0.01 (by Student t test). B, treatment with DHEA inhibited mass formation of U-266 cells. U-266 cells were injected as described in A, and after 12 weeks, mice were sacrificed and mass formations in the peritoneal cavity were observed.

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The Effect of DHEA on Myeloma Cells
Discussion
In this paper, we first show that serum DHEA-S levels are significantly decreased prior to treatment in patients with overt myeloma and some cases of MGUS, and that DHEA as well as DHEA-S can directly inhibit both the proliferation of myeloma cells and the production of IL-6 from BMMNCs from patients with myeloma. Also, the decrease in serum DHEA-S level was apparently correlated with an increase in bone marrow IL-6 levels, as well as with an increase in the fraction of MPC-1 immature myeloma cells in the bone marrow and with an increase in serum CRP level. It is also intriguing that serum DHEA-S levels were decreased in some MGUS cases (precancer state of myelomas), but not in others. In MGUS cases with a significant decrease in serum DHEA-S, the proportions of MPC-1/C0 immature monoclonal plasma cells were significantly increased compared with those in the MGUS cases without a significant decrease in serum DHEA-S levels. However, it remains to be clarified whether or not progression of MGUS to overt myeloma could be triggered by a significant decrease in DHEA-S levels and a significant increase in these immature monoclonal plasma cells.

As previously reported, we also confirmed that DHEA-S could directly inhibit both the proliferation of myeloma cells and the production of IL-6 from BMMNCs from patients with myeloma. Also, the decrease in serum DHEA-S level was apparently correlated with an increase in bone marrow IL-6 levels, as well as with an increase in the fraction of MPC-1 immature myeloma cells in the bone marrow and with an increase in serum CRP level. It is also intriguing that serum DHEA-S levels were decreased in some MGUS cases (precancer state of myelomas), but not in others. In MGUS cases with a significant decrease in serum DHEA-S, the proportions of MPC-1 immature monoclonal plasma cells were significantly increased compared with those in the MGUS cases without a significant decrease in serum DHEA-S levels. However, it remains to be clarified whether or not progression of MGUS to overt myeloma could be triggered by a significant decrease in DHEA-S levels and a significant increase in these immature monoclonal plasma cells.

Also, we confirmed the age-associated decrease in serum DHEA-S levels in male and female subjects. As previously reported, we also confirmed that DHEA-S could...
suppress the production of IL-6. DHEA-S induced the augmentation of PPAR\(\beta\) mRNA expression (25, 26) followed by the increase in 1\textit{A}2\textit{X} gene expression as shown in Fig. 5A and B. Our data also supported that DHEA could not activate NF-\(\kappa\)B in PPAR\(\alpha\) null mice as reported by Poynter and Darnes (27). These may result in the suppression of NF-\(\kappa\)B activity, which could inhibit the production of IL-6 from the BMMNCs in patients with myeloma or bone marrow stromal cell line, KM-102 cells. DHEA-S also suppressed proliferation of myeloma cells \textit{in vivo} and \textit{in vitro}, and it is especially remarkable that implantation of the human myeloma cell line U-266 into SCID-hIL6 transgenic mice was almost completely blocked by simultaneous injection of DHEA. There are two possible explanations for the suppressive effect of DHEA-S on myeloma cell proliferation: one is its inhibition of IL-6 production from myeloma cells such as the IL-6-producing U-266 cell line, and the other is its direct inhibition of myeloma cell proliferation. It is possible that DHEA-induced PPAR\(\beta\) activation in these myeloma cells could induce cell cycle arrest or apoptosis possibly via down-regulation of NF-\(\kappa\)B activity (28). This may be supported by the data that PPAR agonists could directly inhibit the proliferation of myeloma cell line, U-266 cells. Our data also suggest that DHEA-S showed a more suppressive effect on MPC-1\(-\)immature myeloma cells from patients with myeloma as the cases of MM1 and MM3 shown in Fig. 3B. These data could contribute to the therapeutic application of clinical supplementation of DHEA or of PPAR agonists.

The age-associated decline in the production of DHEA may be linked biochemically to the decrease in adrenal P-450csc and/or P-450 17\(\alpha\) 17,20-lyse activity (18). Because the levels of serum cortisol in overt myelomas were not different from those in healthy subjects (data not shown), the decrease in P-450 17\(\alpha\) 17,20-lyse activity could be responsible for the decline in production of DHEA. However, recent reports have shown that skin (29) and renal organs (30) could produce these DHEA and DHEA-S hormones because of the presence of P-450csc and P-450 17\(\alpha\) 17,20-lyse activity (31). Thus, serum levels of these DHEA and DHEA-S are considered to be regulated in more complex ways than expected, and it remains to be clarified how production of DHEA is regulated in the adrenal gland or other responsible organs. Also, increased IL-6 activities could inhibit the activity of enzymes such as P-450csc and P-450 17\(\alpha\) 17,20-lyse activity, which are responsible for DHEA-S production through the hypothalamic-pituitary-adrenal axis, as reported elsewhere (32). We speculate that the regulatory circuit could possibly be working through a decreased DHEA-increased IL-6-more decreased DHEA mechanism, and could be fixed in overt myelomas and some MGUS.

On the other hand, men produce more DHEA-S than women, but women can produce more DHEA from DHEA-S than men, because the gene encoding its steroid sulfatase is located on the X chromosome (33). Furthermore, with regard to race, in healthy men, because the gene encoding its steroid sulfatase is located at the X chromosome (33). Furthermore, with regard to race, in healthy White males (34). This profile in DHEA levels is compatible with I

References

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