Differential biological effects of dehydroepiandrosterone (DHEA) between mouse (B16F10) and human melanoma (BLM) cell lines

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ABSTRACT
Dehydroepiandrosterone (DHEA) is a weak androgen and had been shown to have anti-cancer, anti-adipogenic and anti-inflammatory effects on mouse and other rodent models, but not on humans, suggesting a systemic level difference between mouse and human. Our previous study on DHEA biological functions involving a variety of cell lines, suggested that the functional differences between mouse and human existed even at the cellular level. Hence, using mouse and human melanoma cell models, in-vitro effects of DHEA on cell growth, mechanism of cell death and mechanism of DHEA action were studied. Results indicated a differential biological effects of DHEA between mouse and human melanoma cell lines. These in-vitro studies also suggested that the differential biological effects observed between these two cell lines could be due to the difference in the way DHEA was processed or metabolized inside the cell.

Introduction
Dehydroepiandrosterone (DHEA) is a weak androgen and is an intermediate in testosterone biosynthesis. It is synthesized at a high concentration in adrenal cortex. DHEA level decreases as one ages and this decrease has been attributed to the ailments of old age such as cancer, obesity, heart disease, dementia, mood disorder and immunity. DHEA showed anti-cancer, anti-adipogenic and anti-inflammatory effects in mouse in-vivo experiments. But, these results were not reproduced in human clinical trials. Dr. Nair group reported that no beneficial effect was seen when DHEA was administered to post-menopausal women and aged men, suggesting a systemic level difference between mouse and human. But, a previous study from our lab., using a variety of cell lines showed that the differential effects of DHEA between mouse and human existed not only at the systemic level but also at the cellular level. So, it was postulated that the differences in biological functions between mouse and human could be studied at the cellular level using these two cell lines as model systems. Hence, mouse (B16F10) and human (BLM) melanoma cell lines were used to compare the biological effects of DHEA at the cellular level. Mouse melanoma cell line showed a significant decrease in cell growth, whereas human melanoma cell line showed a muffled effect on cell growth. DHEA induced autophagy in mouse cell line, whereas it induced apoptosis in human cell line to inhibit cell growth. The action of DHEA was mediated through androgen receptor (AR) in mouse cell line, but not in human cell line, suggesting the way DHEA was processed or metabolized inside the cell could be different between these two cell lines. This difference could be responsible for the differential biological actions of DHEA on these two cell lines. This difference in intracellular processing of DHEA could possibly explain the differential biological effects of DHEA previously reported between mouse in-vivo experiments and human clinical trials.
Results

Comparison of dose-curves between mouse and human melanoma cell lines

Based on the result of the previous study,\textsuperscript{10} it was decided to check the dose-response of mouse and human melanoma cell lines to various concentrations of DHEA. Mouse melanoma cells showed a dose-dependent decrease in cell growth (Fig. 1A) from 10 \( \mu \text{M} \) onwards, whereas human melanoma cells showed a muffled effect on cell growth (Fig. 1B). When both cell lines dose-curves were compared (Fig. 1C), the difference in response between these two cell lines appeared at 50 \( \mu \text{M} \) concentration of DHEA treatment. There was a significant decrease (30\%) in mouse melanoma cell growth at 200 \( \mu \text{M} \) concentration of DHEA. Whereas, human melanoma cell line showed a mild decrease (69\%) in cell growth even at 200 \( \mu \text{M} \) concentration of DHEA, suggesting a differential biological effect of DHEA between these two cell lines. Since, there was a difference in DHEA dose-response between these two cell lines, the mechanism of inhibition of cell growth was investigated separately.

Mechanism of inhibition of mouse melanoma cell growth

Necrosis: Initially necrosis was checked as the cause of cell death in mouse cell line. Necrosis was checked by incubating cells with 0.4\% trypan blue for 5 min. Only dead cells would take up the dye and appear as darkly stained cells under microscope. There was no difference in the number of stained cells between untreated control and DHEA (100, 200 \( \mu \text{M} \)) treated mouse melanoma cells (Fig. 2A). So necrosis was ruled out as the mechanism of inhibition of cell growth.

Apoptosis: Apoptosis or programed cell death as the mechanism of cell death was investigated. Since, cell death occurs step by step in apoptosis,
Figure 2. Mechanism of mouse melanoma cell growth inhibition: DHEA treatment resulted in the inhibition of cell growth. The mechanism of inhibition of cell growth was investigated. (A) Necrosis: Necrosis as the cause of cell death was checked first, using 0.4% trypan blue dye. Dead cells would take up the dye and appear as purple colored cells under microscope. There was no difference in the number of stained (arrows point stained cells) cells between control and DHEA (100 μM, 200 μM) treated cells, suggesting necrosis was not the mechanism of cell death. (B) Apoptosis: Apoptosis or programmed cell death as the mechanism was checked initially by staining the cells with DAPI (a fluorescent probe, which specifically stains nucleus) for change in nuclear shape due to condensation of chromatin. The nuclei were circular or oval shaped both in the control and DHEA treated cells, indicating no change in the shape of nucleus after DHEA treatment. (C) Agarose gel electrophoresis: DNA was harvested from untreated control and DHEA 100 μM treated cells. Fifteen μgm of DNA was loaded on 1.2% agarose gel to check for DNA ladder formation. There was no difference in the DNA pattern between control and DHEA 100 μM treated cells, suggesting apoptosis was not the mechanism of cell death. (D) Autophagy: Finally autophagy as the mechanism of cell death was checked by co-incubating cells with DHEA + 3-methyl adenine (0.25 mM). Three methyl adenine was able to partially rescue cell growth in DHEA 100 and 200 μM treated cells compared to plain DHEA treated cells.
initially by condensation of chromosomes leading to a change in nuclear shape. DAPI staining was used to check for change in nuclear shape. DAPI is a fluorescent dye which binds exclusively to the nucleus. After DAPI staining, mouse melanoma cells were viewed under bright and fluorescent light. There was no difference in the shape of nuclei between untreated control and DHEA (100 and 200 μM) treated cells (Fig. 2B). As a follow up study, DNA was harvested from control (untreated) and DHEA (100 μM) treated cells. Fifteen microgram of DNA was loaded on to 1.2% agarose gel and allowed to run for of the gel. The gel was viewed under UV light. There was no DNA ladder formation. Both control cell and DHEA treated cell DNA patterns looked alike (Fig. 2C), suggesting apoptosis was not the mechanism of cell death in mouse melanoma cells.

Autophagy: Autophagy is a process, where the cell devour itself resulting in cell death. As mentioned in materials and methods, 3-MA had been used in various studies to check autophagy or to inhibit autophagy by blocking autophagosome/lysosomal degradation. So, cells were incubated with 100 and 200 μM concentrations of DHEA or DHEA + 0.25 mM of 3-MA. After 48 hrs of incubation, cell growth was monitored by MTT assay. There was a difference in cell growth between plain DHEA and DHEA + 3-MA co-incubated cells (Fig. 3D), with 3-MA co-incubated cells showing a partial increase in cell growth. This partial rescue in cell growth was due to inhibition of autophagy by 3-MA, suggesting the mechanism of inhibition of cell growth was due to autophagy.

**Mechanism of inhibition of human melanoma cell growth**

The mechanism of inhibition of human melanoma cell growth by DHEA was investigated separately.

**Autophagy:** As mouse melanoma cell growth was inhibited by autophagy, it was decided to check autophagy first, as the mechanism of human melanoma cell death. Human melanoma cells were incubated with either plain DHEA or DHEA + 3-MA (2 mM). Concentration of 3-MA was decided by trial and error and was used at 2 mM concentration in earlier studies. There was no difference in cell growth (p > 0.33 at 200 μM) between DHEA or DHEA + 3-MA treated cells (Fig. 3A), suggesting autophagy was not the mechanism of inhibition of human melanoma cell growth.

**Necrosis:** Necrosis as a possible mechanism of cell death was checked by staining with 0.4% trypan blue dye. There was no difference in the number of stained cells between untreated control and DHEA (10, 100, 200 μM) treated cells (Fig. 3B), suggesting necrosis was not the mechanism of cell death.

**Apoptosis:** Finally, apoptosis as the mechanism of cell death was checked. As usual, cell nuclei were initially stained with DAPI to check for change in the shape of nuclei after treatment with DHEA. DAPI stain showed sickle-shaped or crescent-moon shaped nuclei in DHEA (10, 100 and 200 μM) treated cells, compared to oval or circular shaped nuclei in untreated control cells (Fig. 3C). DAPI stain indicated that the nuclei shape were changed in DHEA treated cells compared to control cells nuclei, suggesting apoptosis could be the mechanism of cell death. In order to further confirm apoptosis, DNA was harvested from untreated control and DHEA 10 μM treated cells. Fifteen microgram of DNA was loaded on to 1.2% agarose gel. When the indicator dye ran for of the gel, the gel was removed and viewed under UV light and photographed. DNA ladder containing multiple bands were seen in 10 μM treated cells (Fig. 3D), compared to untreated control cells, where only a single big band was seen. Presence of DNA ladder in DHEA treated sample suggested that the mechanism of inhibition of human melanoma cell growth was due to apoptosis. As another way of confirming apoptosis, cells were incubated either with plain DHEA or DHEA + pan-caspase inhibitor (10 μM). The concentration of caspase inhibitor was decided by trial and error and was used at that concentration in previous paper. Complete rescue in cell growth was seen only at DHEA 10 μM plus caspase inhibitor 10 μM treated cells (Fig. 3E). But, such complete or partial rescue of cell growth was not seen at DHEA 100 μM or 200 μM treated cells. This could be explained easily, as DHEA was at 10 or 20 times higher in concentration at these cells compared to caspase inhibitor (10 μM) concentration. So, there was no effective inhibition of caspase activity by pan caspase inhibitor in the presence of high (100 and 200 μM) concentrations of DHEA.

**Mechanism of DHEA action**

Since, there was a difference in the mechanism of cell death between mouse and human melanoma cell lines, the study of the mechanism of DHEA action became...
Figure 3. Mechanism of inhibition of human melanoma (BLM) cell growth: (A) Autophagy: Since, mouse melanoma cell growth was inhibited by autophagy, it was decided to check autophagy first, as the cause of human melanoma cell death. Cells were co-incubated with DHEA or DHEA + 3-MA 2 mM (concentration of 3-MA was decided by trial and error (12)). After 48 hrs of incubation, cell growth was assessed by MTT assay. There was no difference in cell growth between plain DHEA treated or DHEA + 3-MA co-incubated cells, suggesting autophagy was not the mechanism of cell death. (B) Necrosis: Necrosis as the possible mechanism of inhibition of cell growth was checked by staining cells with 0.4% trypan blue. There was no difference in the number of stained cells (arrows point stained cells) between control and DHEA (10, 100 and 200 μM) treated cells, suggesting necrosis was not the mechanism of cell death. (C) Apoptosis: Apoptosis as the mechanism of cell death was checked first by staining cells with DAPI for change in nuclear shape. DAPI staining showed DHEA treated cells with sickle or crescent-moon shaped nuclei compared to circular or oval shaped nuclei of untreated control cells, suggesting apoptosis could be the mechanism of cell death. (D) DNA agarose gel electrophoresis: DNA was isolated from control and DHEA (10 μM) treated cells to check for DNA ladder formation in DHEA treated cells. Fifteen microgram of DNA was loaded on the gel and ran at 100 volts. The gel was stained with ethidium bromide and viewed under UV light. DHEA treated cells showed DNA ladder formation compared to untreated control cell DNA, suggesting apoptosis was the mechanism of cell death in human melanoma cells. (E) Incubation with pan-caspase inhibitor: In order to further confirm apoptosis, human melanoma cells were incubated with DHEA 10, 100 and 200 μM concentrations either alone or with 10 μM concentration of pan-caspase inhibitor (as higher concentration of caspase inhibitor inhibited cell growth by itself). As expected inhibition of caspase by caspase inhibitor (10 μM) along with DHEA 10 μM showed a complete recovery of cell growth. However, such complete or partial recovery of cell growth was not seen at 100 and 200 μM concentrations of DHEA. This could be explained because DHEA was at 10 and 20 times higher concentration when compared to pan caspase inhibitor concentration (10 μM) in these samples. So pan caspase inhibitor could not inhibit caspase activity at these high concentrations of DHEA.
important. So, it was decided to answer the question, whether DHEA action was mediated through androgen receptor in both the cell lines. Androgen receptor (AR) antagonist bicalutamide was used to block AR. The mechanism of action study was carried out in two ways. First by co-incubating AR antagonist bicalutamide (10 μM) with DHEA at 1, 10, 100 μM concentrations for 48 hrs. There was an increase in cell growth at all the concentrations of DHEA + Bicalutamide co-incubated cells compared to DHEA alone incubated cells, indicating there was a competition between DHEA and bicalutamide for AR. Secondly, mouse melanoma cells were pre-incubated with bicalutamide (10 μM) for 60 – 90 min and later DHEA was added. Mouse melanoma Cells showed an increase in cell growth at all the concentrations of DHEA treatment after bicalutamide pre-incubation compared to straight DHEA treatment, suggesting the action of DHEA was mediated through AR.

in the bicalutamide pre-incubated experiment, mouse melanoma cell growth was increased at all the concentrations of DHEA treatment (Fig. 4B). Same type of co-incubation of DHEA with bicalutamide and pre-incubation with bicalutamide, followed by DHEA treatment were carried out with human melanoma cells. There was no difference in cell growth in DHEA + bicalutamide co-incubated cells compared to plain DHEA treated cells (Fig. 5A), suggesting there was no direct competition between DHEA and bicalutamide for AR. Again there was no difference in cell growth in pre-incubated experiment between plain DHEA treated cells and bicalutamide pre-treated cells followed by DHEA treated cells (Fig. 5B), suggesting the
action of DHEA was not mediated through AR in human melanoma cells.

**Discussion**

DHEA is known as a weak androgen. Even though it is called an androgen, it is present in high concentration in male and female. So, DHEA function is still unclear. DHEA level decreases as one ages. This decrease in DHEA level had been attributed to various diseases seen in elderly people including cancer. Clinical trials with DHEA were not successful,\(^7\)–\(^9\) as they did not reproduce any biological effect seen in small animals such as mouse and rat. This was explained initially that mouse had low levels of DHEA, whereas human had high DHEA level, so the effect was not detectable in humans due to desensitization of receptor. However, our earlier study\(^10\) using a variety mouse and human cell lines showed that the difference existed even at the cellular level and so cell lines could be used to study the differential biological actions of DHEA. Hence, present study was carried out using mouse (B16F10) and human melanoma (BLM) cell lines to check the differential biological effects of DHEA.

1) Dose-response curve: As anticipated, there was a difference in dose-response curves between mouse and human melanoma cell lines. Mouse melanoma cells showed a dose-dependent decrease in cell growth and significant inhibition at 100 and 200 \(\mu M\) concentrations of DHEA, whereas human melanoma cell line did not show such dose-dependent decrease in cell growth. Only mild inhibition in cell growth was seen even at 200 \(\mu M\) concentration of DHEA.

2) Mechanism of Inhibition of cell growth: The mechanism of inhibition of mouse melanoma cell growth was due to autophagy, whereas human melanoma cell growth inhibition was due to apoptosis.

3) Mechanism of DHEA action: The mechanism of DHEA action was mediated through androgen receptor in mouse melanoma cell line. But DHEA action was not mediated through AR in human melanoma cell line. Though DHEA action could be mediated through special receptor or even through estrogen receptor in human cell line, but these receptors were not checked. Only androgen receptor was checked because several papers cited the conversion of DHEA into testosterone in peripheral tissues\(^22\)–\(^24\) and skin being a peripheral site, only AR was checked. In fact in mouse cell line, DHEA action was mediated through AR.

Above *in-vitro* experiment suggested that the differences in biological actions of DHEA between the 2 cell lines could be due to the difference in the way DHEA was processed or metabolized inside the cell. Again as suggested in our previous paper,\(^10\) present paper with DHEA underscored the idea that the biological differences between mouse and human could be studied at the cellular level itself, instead of a systemic level model.

**Conclusion**

The way DHEA was processed or metabolized inside mouse and human melanoma cells could be responsible for the differences in biological effects observed between mouse (B16F10) and human melanoma (BLM) cell lines. This difference in the processing of DHEA inside the cell could possibly explain the biological differences observed between mouse *in-vivo* experiments and human clinical trials. This kind of selective response of cells to a hormone had already been reported and was shown to be due to the intrinsic difference in the molecular apparatus for response to sex steroids (progesterone and estrogen). This observation with respect to females suffering from premenstrual dysphoric disorder (PMDD), but not in other females was already published in Journal of Molecular Psychiatry.\(^26\) This publication lent support to our finding with respect to DHEA action on mouse cell line mediated through AR, but not in human melanoma cell line.

**Materials and methods**

Dehydroepiandrosterone (DHEA), Pan-caspase inhibitor, bicalutamide were all obtained from Sigma Chemical Company, St. Louis, MO. MTT (3-\([4,5\)-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), DAPI (4’-6 diamidino-2-phenylindole), trypan blue, isopropanol, 3-methyladenine, agarose were also obtained from Sigma Chemical Company. Fetal bovine serum (FBS), Trypsin-EDTA (1X), and PBS powder were purchased from Atlanta biologicals, Lawrenceville, GA. RPMI and antibiotic/antimycotic solution 100X (10,000 I.U/ml penicillin, 10 mg/ml streptomycin, 25 \(\mu g/ml\) amphotericin B) were purchased from Fisher scientific, Houston, TX. SLT
Spectra plate reader was used for quantitation of MTT proliferation assay.

Growth Medium (GM): All cell culture works were carried out in RPMI 1640 medium containing 10% FBS +1X Pen/Strep/Ampho, as mentioned in our previous papers. Cell growth was quantitated using MTT proliferation assay.

MTT proliferation assay: Mouse melanoma (B16F10) and human melanoma (BLM) cells were suspended in growth medium (GM) and plated at a density of 1 × 10^4 cells/well in a costar 96 well plate. Cells were left overnight at 37°C to attach to the plate. Following day, growth medium was replaced by GM containing hormone at different concentrations and incubated for 48 hrs. After 48 hrs medium was replaced by 100 μl of 1 in 10 diluted (in GM) MTT solution and incubated for another 4 hrs at 37°C. After 4 hrs MTT solution was removed. MTT was reduced by metabolically viable cells to a purple colored water insoluble for-macy of resultant purple color was measured at 570 nm in a SLT spectra plate reader.

Trypan blue staining: Trypan blue is a dye that does not interact with cell unless the cell membrane is damaged. Healthy, undamaged cells exclude the dye, but it is readily taken-up by damaged cells and renders them clearly visible under microscope. After 48 hrs of incubation of cells with DHEA, 100 μl of 0.4% trypan blue in PBS was added to the chamber slide containing 1 × 10^4 cells and incubated for 5 min. at room temperature. Cells were washed with PBS to remove excess trypan blue dye and then examined under microscope. Only dead cells would appear as darkly stained cells under microscope.

DAPI staining: DAPI is a blue fluorescent probe. DAPI stains nucleus specifically, with little or no cytoplasmic labeling. After 48 hrs of incubation of cells with hormone, cells in the chamber slide were fixed with 2% paraformaldehyde for 20 min. Cells were washed with PBS to remove paraformaldehyde. One hundred microliter of 1 in 1000 diluted DAPI was added to the cells and incubated in the dark for 5 min. Excess DAPI was removed by washing with PBS and cells were viewed under bright and fluorescent light microscope.

Agarose gel electrophoresis: 1.2% agarose gel was made by boiling agarose in 1X TAE buffer. After cooling the solution, ethidium bromide was added to the solution and poured into the gel apparatus. Fifteen microgram of DNA was loaded on to the well and ran at 100 volts till the bromophenol dye moved 3/4 of the gel. The gel was photographed under UV light.

Co-incubation with 3-methyladenine (3-MA): 3-methyladenine specifically inhibited formation of autophagosome in cells. Three-methyladenine (3-MA) had been used in study to detect autophagy or to inhibit autophagy. In fact 3-MA had been used in human hepatoma cell line (HepG2) treated with DHEA to check autophagy. So, cells were incubated either with DHEA or DHEA + 3-MA to check the effect on cell growth, as monitored by MTT assay.

Co-incubation and pre-incubation of cells with bicalutamide: Bicalutamide is an androgen receptor antagonist and prevents androgens such as testosterone (T), DHEA and AD (androstenedione) from binding to androgen receptor (AR). This function of bicalutamide was made use of to determine the ability of DHEA to bind to AR. Initially, DHEA and bicalutamide were co-incubated for 48 hrs to check whether DHEA action on cell growth was blocked or increased or decreased by bicalutamide competing with DHEA for AR. Secondly, bicalutamide alone was incubated with the cells for 60 – 90 min (pre-incubated) before adding DHEA. The reason for the later addition of DHEA to the cells, was to check the ability of DHEA to bind to androgen receptor to inhibit cell growth after the receptors were blocked by bicalutamide pre-incubation.

Statistical analysis: All experimental points were carried out in triplicate (3 wells). Each experiment was repeated a minimum of three times to check for consistency in results. Each experimental point was expressed as Mean ± SEM. Significance between any two experimental conditions was decided using Student’s ‘t’ test. Unpaired t-test was used to determine the p value and a p value of 0.05 or less was considered significant.

Abbreviations

AD Androstenedione; 3-MA – 3-methyl adenine
AR Androgen receptor; DAPI – (4′-6 diamido-2-phenylindole)
Bic. Bicalutamide
DHEA  Dehydroepiandrosterone; MTT – (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
PMDD  Premenstrual dysphoric disorder;
T. Testosterone

Conflict of interest
Authors have nothing to disclose.

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