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DHEA inhibits cell growth and induces apoptosis in BV-2 cells and the effects are inversely associated with glucose concentration in the medium

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Abstract

Dehydroepiandrosterone (DHEA), a major steroid secreted by the adrenal gland which decreases with age after adolescence, is available as a nutritional supplement. DHEA is known to have antiproliferative effects but the mechanism is unclear. In this study using BV-2 cells, a murine microglial cell line, we investigated the effect of DHEA on cell viability and the interaction between DHEA and glucose concentrations in the medium. We showed that DHEA inhibited cell viability and G6PD activity in a dose-dependent manner and that the effect of DHEA on cell viability was inversely associated with glucose concentrations in the medium, i.e. lowered glucose strongly enhanced the inhibition of cell viability by DHEA. DHEA inhibited cell growth by causing cell cycle arrest primarily in the G0–G1 phase, and the effect was more pronounced at zero glucose (no glucose added, G0) than high glucose (4.5 mg/ml of the medium, G4.5). Glucose deprivation also enhanced apoptosis induced by DHEA. At G4.5, DHEA did not induce formation of DNA ladder until it reached 200 μM. However, at G0, 100 μM DHEA was able to induce apoptosis, as evidenced by the formation of DNA ladder, elevation of histone-associated DNA fragmentation and increase in cells positively stained with annexin V-FITC and annexin V-FITC/propidium iodide. The interactions between DHEA and glucose support the contention that DHEA exerts its antiproliferative effects through alteration of glucose metabolism, possibly by inhibition of G6PD activity leading to decreased supply of ribose-5-phosphate for synthesis of DNA and RNA. Although DHEA is only antiproliferative at pharmacological levels, our results indicate that its antiproliferative effect can be enhanced by limiting the supply of glucose such as by energy restriction. In addition, the present study shows that glucose concentration is an important factor to consider when studying the antiproliferative and toxicological effects of DHEA.

Keywords: DHEA; Steroid; Tumor

1. Introduction

Dehydroepiandrosterone (DHEA), a major steroid secreted by the adrenal gland [1], is available as a nutritional supplement in some countries (e.g. USA). The secretion of DHEA in humans reaches maximum at ~25 years of age, and the serum DHEA concentration decreases afterwards, with ~10% remaining at the age of 70 or 80 [1,2]. Moreover, DHEA has been reported to possess several beneficial effects, including antiobesity [3,4], hypoglycemia [4–7], anticancer [8–11], anti-atherosclerosis [12,13], and preventing brain from aging [14]. However, the mechanisms of these effects remain unclear [3,4,15].

DHEA has been reported to have antiproliferative effect in animal tumor models and malignant cell lines

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[16–23]. This effect of DHEA has been attributed to its inhibition of G6PD activity, with subsequent blockage of NADPH and ribose-5-phosphate formation, both of which are essential for cell growth [8,16–19,24]. However, the role of G6PD inhibition in the antiproliferative effect of DHEA on human breast cancer cells [20,21] and airway smooth muscle cells [22] has been questioned or even excluded. In addition to G6PD inhibition, DHEA can also affect the activity of several enzymes of the glycolysis and glucoenogensis pathways [4,25,26]. In animal studies, DHEA has been reported to have a hypoglycemic effect [4–7]. The increase of glucose uptake was observed in many cell lines incubated with DHEA [27–29]. Brignardello et al. [30] reported that DHEA protects bovine retinal capillary pericytes against glucose toxicity. Results from all these studies suggest that at least some of the effect of DHEA, such as on cell growth, is closely related to glucose metabolism.

In addition to its antiproliferative effects, DHEA has been reported to have neuroprotective properties (e.g. against oxidative damage to hippocampal cells) [31–33]. Because microglial cells have been implicated in the pathogenesis of numerous neurodegenerative diseases [34], we employed BV-2 cells, a murine microglial cell line transfected with the v-raf/v-myc oncogene [35], to study the antiproliferative effect of DHEA and its interaction with glucose. Our working hypothesis was that the effect of DHEA on cell growth would be affected by glucose concentration in the medium. Here, we report some novel findings that in addition to inhibiting cell growth, DHEA induced apoptosis of BV-2 cells and that these effects of DHEA were inversely associated with glucose concentration in the medium.

2. Materials and methods

2.1. Cell culture and incubation

The murine BV-2 cell line developed by Dr V. Bocchini (University of Perugia, Perugia, Italy) was a gift from Dr J. S. Hong (NIEHS, NIH, NC). Immortalization of the BV-2 cell line by infecting primary microglial cell cultures with a v-raf/v-myc oncogene carrying retrovirus (J2) has been described previously [35]. Cells were cultured in high glucose (4.5 mg/ml) Dulbecco's modified Eagle medium (DMEM, Gibco) with 10% (FBS) at 37°C in a humidified incubator under 5% CO_2 and 95% air. For incubation with DHEA, the medium was changed to basal DMEM (Gibco, without glucose, pyruvate and phenol red, etc.) with 10% FBS. DHEA (10 or 20 mM) was prepared in ethanol; the final concentrations of ethanol were kept $< 1\%$, at which level ethanol did not significantly affect any of the assays described below.

2.2. Cell viability assay

Cell viability was assayed using a modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric assay [36]. The cells were cultured in 12-well plates with 5×10^4 cells per well. After incubation with DHEA for 48 h, the cultured medium was aspirated, and 0.5 ml of 300 μg MTT/ml of DMEM medium was added. After incubation for 1 h, the medium was aspirated, and the formazan was extracted in 0.5 ml of DMSO. A volume (100 μl) of the extract was transferred into a 96-well microplate, and the absorbance was measured at 540 nm.

2.3. Measurement of G6PD activity

G6PD activity was determined in the cell extract as described by Lee et al. [37] and Glock et al. [38]. Nearly confluent cell cultures were washed in PBS, centrifuged (200***g*** for 10 min at 4°C), suspended in PBS ($\sim 2 \times 10^7$ cells/ml), and transferred into a microcentrifuge tube followed by sonication (six times, 10-s burst with 1-min intervals). Clear extracts were obtained by centrifugation at 12 000***g*** for 20 min at 4°C and kept on ice before assay. A 200-μl cell extract with or without 4.5 mg glucose/ml was mixed with 0.75 ml of 0.05 M Tris-HCl buffer containing 3 mM $MgCl_2$ (pH 7.8), 25 μl of 8 mM $NADP^+$ and 25 μl of 0.13 M G6P. Activity was measured as the change in absorbency at 340 nm.

2.4. Cell cycle analysis

Cell cycle analysis was carried out as described by Yoshizumi et al. [39]. After plating $\sim 5 \times 10^5$ cells onto a 60-mm culture dish, the cells were cultured overnight for attachment. After incubation with DHEA for 24 h, the cells were washed twice in PBS, digested with trypsin-EDTA (TE) buffer, suspended in 3 ml of PBS, followed by centrifugation. Cell pellets collected in 1 ml of PBS were fixed in 3 ml of 100% ethanol for 1 h and then added 0.5 ml of 0.05% RNase in PBS containing 0.5% Triton X-100. After digestion at 37°C for 30 min, 0.5 ml of 50 μg propidium iodide (PI)/ml was added. After standing at 4°C for 30 min, the suspensions were filtered, and the cell cycle was analyzed using a flow cytometer (FACSCalibur). The data were analyzed using MODFIT cell cycle analysis program.

2.5. DNA ladder detection

BV-2 cells (2×10^6 cells) were plated onto 100-mm dishes and cultured until confluency. After incubation with DHEA for 10 h, floating cells were collected, and the DNA was isolated using the DNeasy Tissue Kit (Qiagen, Santa Clarita, CA). An aliquot (10 μl) of the DNA was mixed with $6 \times$ loading buffer and loaded

onto a 1.8% agarose gel in 0.5 × TBE buffer. The gel was stained with ethidium bromide (0.5 μg/ml) and photographed (Polaroid, Fotodyne). A 100-bp DNA ladder (Parmacia Biotech, Uppsala, Sweden) was included as standard.

2.6. Histone-associated DNA fragmentation test

The histone-associated DNA fragmentation was assayed for cell death using ELISA PLUS kit (Roche, Mannheim, Germany). Briefly, $\sim 1 \times 10^4$ cells were plated onto a 96-well microplate. After incubation with DHEA for 24 h, cells were centrifuged (200***g***, 10 min) and lysed using lysis buffer provided by the kit. The lysate (50 μl/well) was re-centrifuged and transferred into microplates coated with streptavidin. After incubation with the immunoreagent containing anti-histone-biotin and anti-DNA-POD for 2 h at room temperature, the substrate solution was added, and the absorbance at 405 nm was measured.

2.7. Apoptosis detection by staining with annexin V-FITC and propidium iodide

The Annexin V-FITC Apoptosis Detection kits I (Pharmingen, San Diego, CA) was used for detection of apoptosis. BV-2 cells (1×10^6 cells) were plated onto a 60-mm dish and cultured overnight, followed by incubation with DHEA for 24 h. Cells were harvested by TE digestion, washed twice in cold PBS (1.4 M NaCl, 27 mM KCl, 100 mM KH_2PO_4/K_2HPO_4, pH 7.2), suspended in 1 × binding buffer at $\sim 1 \times 10^6$ cells/ml, and 0.1 ml of the cells was transferred to a 5-ml culture tube. The cells were then stained with 5 μl of Annexin V-FITC (which binds to exposed membrane phosphatidylserine of early apoptotic cells) and 2 μl of propidium iodide (PI, a standard flow cytometric probe used to distinguish viable cells from nonviable cells) at a concentration of 50 μg/ml. After standing for 15 min in the dark, 400 μl of 1 × binding buffer was added to each tube, and the cells were analyzed by flow cytometry immediately.

2.8. Statistical analysis

Data were analyzed by analysis of variance (ANOVA) followed by LSD (least significant difference) for group mean comparison using SPSS program. P values < 0.05 were considered significant.

3. Results

3.1. Effect of glucose and DHEA concentrations on cell viability

Fig. 1 shows the viability of BV-2 cells incubated at 37°C for 48 h with 0, 25, 50 and 100 μM DHEA and 0 (G0), 0.56 (G0.56), 1.1 (G1.1) and 4.5 (G4.5) mg glucose/ml of the medium. At G4.5 (25 mM, the glucose level of the high glucose DMEM medium) and at G1.1 (6.1 mM, approximately the normal serum glucose level), the inhibition of cell viability was only evident when DHEA reached 100 μM (35 and 58% inhibition, $P < 0.001$, for G4.5 and G1.1, respectively). The inhibition of cell viability by DHEA was enhanced when glucose concentrations decreased, and the effect of glucose was dose-dependent. At G0, DHEA at 25, 50 and 100 μM decreased cell viability by 22% ($P < 0.05$), 55% ($P < 0.001$) and 98% ($P < 0.001$), respectively. The solvent control (i.e. ethanol) showed a slight but dose-dependent decrease in cell viability (Fig. 1). At 1% ethanol, which was the highest final concentration used, cell viability was decreased by only 8% ($P > 0.05$).

3.2. Effect of glucose on DHEA-induced inhibition of cell growth and G6PD activity

To avoid apoptosis in cell cycle analysis, we used 0–100 μM DHEA at G4.5, compared to 0–50 μM DHEA at G0, because 100 μM DHEA at G0 produced DNA ladder (see Section 3.3 for apoptosis). After

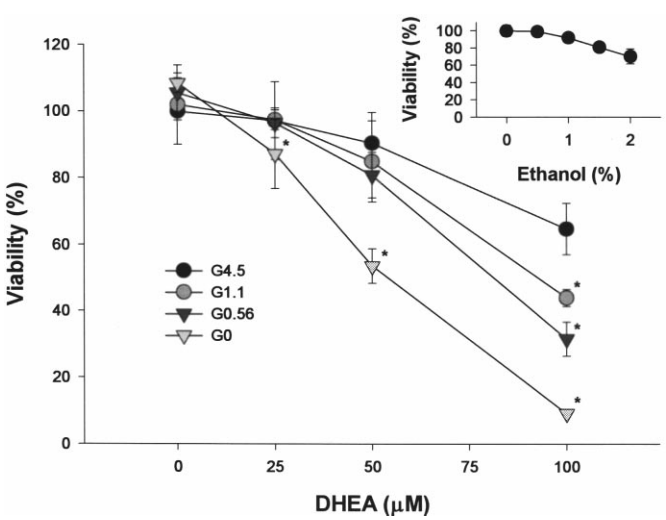

Fig. 1. Effects of glucose and DHEA concentrations on the viability of BV-2 cells. DHEA was added at 0, 25, 50 and 100 μM and glucose was added at 0 (G0), 0.56 (G0.56), 1.1 (G1.1) and 4.5 (G4.5) mg/ml of the medium. Incubation was carried out at 37°C for 48 h. Cell viability was expressed as % of control (i.e. without DHEA but with 4.5 mg glucose/ml). The inset shows the effect of solvent control (i.e. ethanol) on cell viability, which was decreased by 8% ($P > 0.05$) at 1% ethanol, the highest final concentration used (1%). Values (means ± SD of triplicate samples representative of several experiments) with an asterisk (*) are significantly different ($P < 0.05$) from the control (G4.5) at each concentration of DHEA.

Table 1
Cell cycle analysis on BV-2 cells incubated with DHEA at 37° for 24 h without or with 4.5 mg glucose/ml of the medium

Cell cycle stage	G4.5[a]			G0		
	D0[b]	D50	D100	D0	D25	D50
G0–G1	67.7%	72.2%	83.3%	73.5%	73.8%	78.9%
S	25.1%	27.5%	16.5%	23.2%	26.0%	21.2%
G2–M	7.2%	0.4%	0.2%	3.3%	0.1%	0.0%

[a] G4.5 and G0 stand for glucose added to the medium at 4.5 or 0 mg/ml.
[b] D0, D25, D50 and D100 stand for DHEA added to the medium at 0, 25, 50 and 100 μM, respectively.

incubation with 100 μM DHEA at G4.5 for 24 h, cells in the G2-M phase decreased from 7.2 to 0.2%, and those in the G0–G1 phase increased from 67.7 to 83.3%; the DHEA effect was dose-dependent (Table 1). The inhibition of cell growth by DHEA was enhanced at G0, since essentially no BV-2 cells (0.1%) existed in the G2-M phase when DHEA was ≥ 25 μM. Glucose deprivation itself also inhibited cell growth (e.g. cells in the G2-M phase decreased from 7.2 to 3.3%), and the effects of DHEA and glucose were additive.

DHEA inhibited G6PD activity in a dose-dependent manner; 50 and 100 μM DHEA inhibited the activity by 57 ($P < 0.001$) and 74% ($P < 0.001$), respectively (Fig. 2). However, the inhibition of G6PD activity by DHEA was not affected by glucose concentration.

3.3. Effects of glucose concentration on DHEA-induced apoptosis

At G4.5, incubation of cells for 10 h with 200 μM DHEA, but not 100 μM or lower concentrations, induced visible formation of DNA ladder (G4.5, Fig. 3). However, the ladder was already visible at 100 μM DHEA at G0. The histone-associated DNA fragmentation was not significantly affected in BV-2 cells incubated at G4.5 with DHEA up to 100 μM for 24 h (Fig. 4). Glucose deprivation affected the DNA fragmentation only at high DHEA. At G0, the DNA fragmentation of BV-2 cells incubated with 100 μM DHEA for 24 h was increased by 212% ($P < 0.001$).

The dual staining of cells with annexin V-FITC and PI is another method for identifying apoptosis. Cells that stain positive for annexin V-FITC and negative for PI are in the early stage of apoptosis while those stained positive for both annexin V-FITC and PI are in the later stages of apoptosis or are already dead. At G4.5, incubation with 100 μM DHEA for 24 h increased the annexin V-FITC positive cells (lower right quadrant) and the annexin V-FITC/PI positive cells (upper right quadrant) from 2.1 to 8.8% and from 11.1 to 16.9%, respectively (Fig. 5(a–b)). At G0, the effect of DHEA was strongly enhanced; the annexin V-FITC positive cells and the annexin V-FITC/PI positive cells increased from 0.9 to 31.9% and from 14.9 to 22.6%, respectively (Fig. 5(c–d)).

4. Discussion

The present study investigated the effect of DHEA on cell viability and apoptosis and the interaction with glucose in BV-2 cells. We demonstrated that DHEA inhibited cell viability in a dose-dependent manner and that this effect of DHEA was inversely associated with glucose concentrations in the medium, i.e. lowered glucose strongly enhanced the inhibition of cell viability by DHEA. DHEA has been shown to induce apoptosis in vivo [40,41] but not in vitro [40]. Here, we showed that DHEA induced apoptosis only at very high level (200 μM DHEA at G4.5) and that the effect of DHEA was enhanced by glucose deprivation. The results demonstrate that glucose concentration in the medium has strong effects on the biological actions of DHEA. To our knowledge, this is the first report on the direct interaction between glucose concentrations and the antiproliferative effects, including apoptosis, of DHEA.

DHEA has been reported to have chemopreventive effects [8–11]. In cell culture studies, DHEA has consistently been shown to inhibit cell growth [16–23], and DHEA induces growth arrest in the G0–G1 phase in

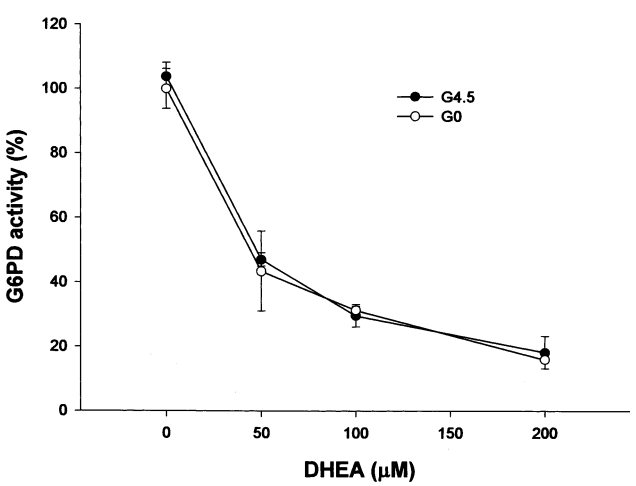

Fig. 2. Effects of glucose and DHEA concentrations on glucose 6-phosphate dehydrogenase (G6PD) activity. G6PD activity was assayed in the cell extract incubated with DHEA with or without addition of 4.5 mg glucose/ml of PBS. Values are means ± SD, $n = 2–3$.

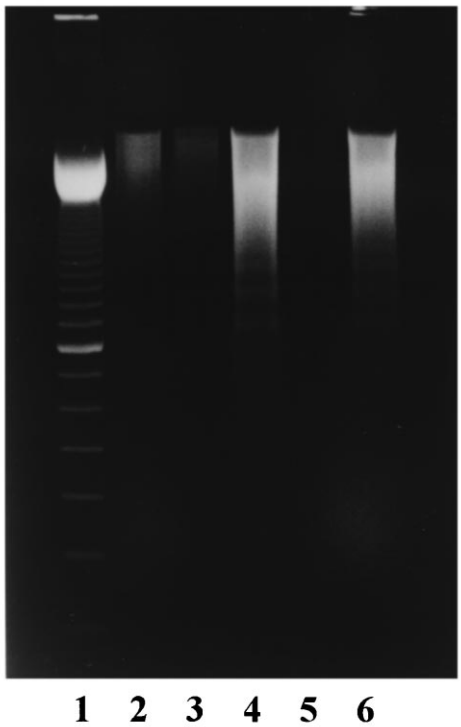

Fig. 3. Effect of glucose and DHEA on the formation of DNA ladder in BV-2 cells incubated at 37°C for 10 h. Lanes from left to right are: (1) A 100-bp ladder maker; (2) control (without DHEA; without glucose); (3) 50 μM DHEA without glucose; (4) 100 μM DHEA without glucose; (5) 100 μM DHEA with 4.5 mg glucose/ml; and (6) 200 μM DHEA with 4.5 mg glucose/ml.

human colonic adenocarcinoma cells [19] and in Ehrlich's tumor cells [42]. Our results from cell cycle analysis on the growth inhibition of BV-2 cells by

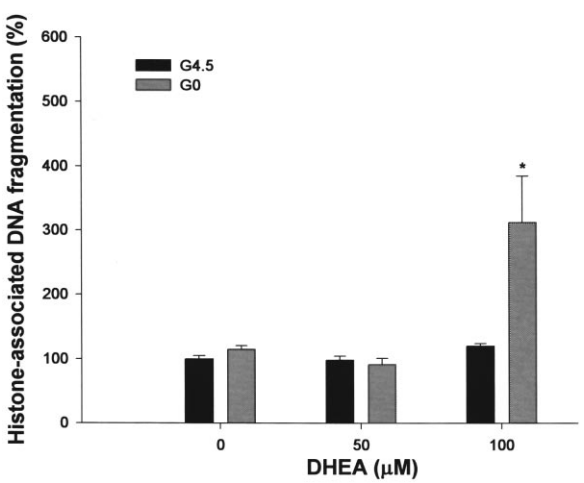

Fig. 4. Effect of glucose and DHEA concentrations on histone-associated DNA fragmentation in BV-2 cells incubated at 37°C for 24 h. DHEA was added at 0, 50 and 100 μM and glucose was added at 0 (G0) and 4.5 (G4.5) mg/ml of the medium. Values (means ± SD, $n = 3$) with an asterisk (*) are significantly different ($P < 0.05$) from the control (G4.5) at each concentration of DHEA.

DHEA are consistent with those obtained from other cell lines. The antiproliferative effect of DHEA has been attributed to its non-competitive inhibition of G6PD [43–45], which results in diminished production of ribose-5-phosphate, leading to decreased biosynthesis of DNA and RNA, and consequently, to inhibition of cell growth [8,17,18,24]. Two lines of evidence from our study support such a mechanism. First, the DHEA concentrations that effectively inhibited G6PD activity were lower than those that inhibited cell viability. For instance, 50 μM DHEA inhibited 57% G6PD activity (Fig. 1, G4.5) but only slightly inhibited cell viability (Fig. 2, G4.5). Second, the DHEA-induced inhibition of cell viability was inversely associated with glucose concentration in the culture medium. In accord with the latter finding, glucose has been shown to regulate cell division of androgen-independent prostate cancer cells [46] and to stimulate cell cycle from the G0–G1 to the S and M phases in vascular smooth muscle cells [47]. In addition, glucose deprivation has been reported to induce cell growth arrest in the G0–G1 phase [46,48], an effect resembling that of DHEA [19,42] and of the glucose anti-metabolite 2-deoxyglucose [48,49]. However, inhibition of G6PD may not be the only or most important mechanism of the antiproliferative effect of DHEA. For instance, DHEA can affect the activity of several enzymes in the glycolysis and glucoenogensis pathways [4,25,26]. In addition, the inhibition of G6PD by DHEA may not account for the growth inhibition of certain types of cells. For example, the inhibitory effect of DHEA on the growth of rat tracheal smooth muscle (a primary cell culture) stimulated with platelet-derived growth factor is not attributed to inhibition of G6PD activity or of cholesterol metabolism, since supplementation of growth medium with ribonucleosides and deoxyribonucleosides does not overcome the effect of DHEA [22]. Rather, DHEA decreases the binding of the transcription factor activator protein-1, a later response important for expression of genes that mediate DNA synthesis and cell cycle progression [22]. Moreover, in vivo studies have not consistently demonstrated the inhibition of G6PD by DHEA [3]; some animal studies [50,51] have even reported an increase in tissue G6PD activity after DHEA treatment.

The ability to induce apoptosis is important in cancer chemoprevention [52–54]. Previously, DHEA has been reported to induce apoptosis only in vivo but not in vitro for blood lymphocyte [40]. DHEA was also found to induce apoptosis in the thymus of p53-defeciency mice [41]. Using several apoptosis assays including the formation of DNA ladder, histone-associated DNA fragmentation and staining with annexin V-FITC and propidium iodide, we found no apoptosis of BV-2 cells incubated with 100 μM DHEA unless the cells were deprived of glucose. Thus, DHEA is unlikely to induce apoptosis at physiological concentrations (i.e. at low

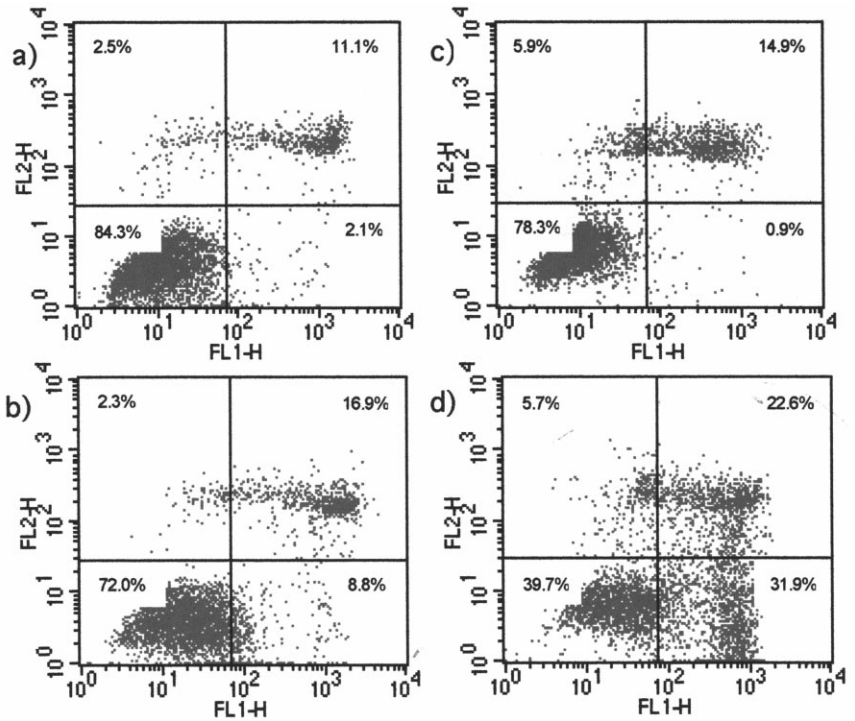

Fig. 5. Effects of glucose (0, 4.5 mg/ml) and DHEA (0, 100 μM) on the growth of BV-2 cells assayed by flow cytometry following dual-staining with annexin V-FITC (FL1-H) and propidium iodide (FL2-H). Incubations were at 37° for 24 h; (a) without DHEA but with glucose; (b) with DHEA and glucose; (c) without DHEA and glucose; (d) with DHEA but without glucose. Results are expressed as % of total stained cells. The annexin V-FITC positive cells are shown at the lower right quadrant and the annexin V-FITC/propidium iodide positive cells are at the upper right quadrant.

micromolar levels, [2]), and our results suggest that DHEA may only be chemopreventive under specific conditions such as glucose depletion. Interestingly, DHEA may also be chemopreventive under energy restriction, since calorie restriction has been shown to increase the rate of apoptosis in the thymus in p53-deficient mice [41]. In these p53-deficient mice, the action of calorie restriction is different from that of DHEA because DHEA treatment resulted in decreased bcl-2 but not Bax mRNA levels in the thymus while the former did not change either bcl-2 or Bax mRNA expression [41].

5. Conclusion

This study in BV-2 cells has demonstrated that DHEA at pharmacological levels decreases cell viability by causing an arrest in the G0–G1 phase and by inducing apoptosis and that these effects of DHEA are strongly enhanced when glucose concentrations were decreased. The interaction between DHEA and glucose supports the contention that DHEA may exert its effect through alteration of glucose metabolism, possibly by inhibition of G6PD leading to decreased supply of ribose-5-phosphate for synthesis of DNA and RNA. Although DHEA is only antiproliferative at pharmacological levels, our results suggest that its effects can be enhanced either by limiting the supply of glucose such as by energy restriction or by the use of other pentose cycle inhibitors to limit the supply of RNA and DNA for cell replication. In this context, Raïs et al. [42] have most recently shown that a combination of oxythiamine, a transketolase inhibitor, with DHEA synergistically inhibits the proliferation of Ehrlich's tumor cells in mice.

Acknowledgements

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