Oxythiamine and dehydroepiandrosterone induce a G_1 phase cycle arrest in Ehrlich's tumor cells through inhibition of the pentose cycle

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Abstract Transketolase (TK) reactions play a crucial role in tumor cell nucleic acid ribose synthesis utilizing glucose carbons, yet, current cancer treatments do not target this central pathway. Experimentally, a dramatic decrease in tumor cell proliferation after the administration of the TK inhibitor oxythiamine (OT) was observed in several in vitro and in vivo tumor models. Here, we demonstrate that pentose cycle (PC) inhibitors, OT and dehydroepiandrosterone (DHEA), efficiently regulate the cell cycle and tumor proliferation processes. Increasing doses of OT or DHEA were administered by daily intraperitoneal injections to Ehrlich's ascites tumor hosting mice for 4 days. The tumor cell number and their cycle phase distribution profile were determined by DNA flow histograms. Tumors showed a dose dependent increase in their G_0-G_1 cell populations after both OT and DHEA treatment and a simultaneous decrease in cells advancing to the S and G_2-M cell cycle phases. This effect of PC inhibitors was significant, OT was more effective than DHEA, both drugs acted synergistically in combination and no signs of direct cell or host toxicity were observed. Direct inhibition of PC reactions causes a G_1 cell cycle arrest similar to that of 2-deoxyglucose treatment. However, no interference with cell energy production and cell toxicity is observed. PC inhibitors, specifically ones targeting TK, introduce a new target site for the development of future cancer therapies to inhibit glucose utilizing pathways selectively for nucleic acid production.

Key words: Cell cycle regulation; Glucose metabolism; Oxythiamine; Dehydroepiandrosterone

1. Introduction

Increased metabolism of glucose in malignant tissue is the most characteristic sign of the severe metabolic imbalance between tumor cells and their surrounding tissue [1]. This phenomenon utilizing the ^{18}fluoro-deoxyglucose tracer molecule, detected by positron emission tomography, has become a reliable technique for detecting and classifying tumors. This technique has been useful in detecting human malignancies [2] based on the common characteristics that malignant tumors accumulate glucose about 8.5–15 times more intensively than their surrounding normal tissue marked by their increased differential glucose uptake ratio.

It is broadly contemplated that glucose carbons intensively accumulate in cancer cells because of increased energy requirements and nucleic acid synthesis and that labelling glucose carbons is a useful diagnostic tracer in cancer. Recent new reports also indicate that increased glucose uptake in malignant tumors will become utilized in the treatment of cancer using 2-deoxyglucose [3]. Interestingly, 2-deoxyglucose, a false substrate of glycolysis and the pentose cycle (PC), induced a significant G_1 cell cycle arrest in leukemia cells and decreased the percentage of cells in S and G_2-M phases. Introduction of apoptosis by combined treatment with recombinant human TNF-α and 2-deoxyglucose has also been achieved in the same model [4]. However, false glucose metabolites such as deoxyglucose fall short of acting selectively on intracellular anabolic reactions by strongly influencing intracellular energy production and basic cell functions other than the cell division process. This significant disadvantage using deoxyglucose is yet to be overcome that affects not only tumors but also all high glucose utilizing tissues such as the brain and testis.

Studies utilizing isotopically labelled glucose carbons recovered from tumor cell RNA and DNA have revealed that glucose carbons are also the main source of de novo lipid [5] and nucleic acid synthesis [6,7] besides their central role in energy production. Therefore, glucose utilizing intracellular anabolic pathways in the PC offers a new approach to tumor therapy [8]. Since the proliferation process is governed by the replication of DNA in the S cell cycle phase known as the 'S peak' in malignant tumors, the intensive non-oxidative synthesis of ribose by transketolase (TK) [6,7] during this process is a promising new target site for cancer treatment protocols. The recently observed high label loss ($>35\%$) on the sixth (C6) carbon of glucose recovered from DNA after culturing H9 (T-cell) and Hep G2 tumor cells for 53 days in the presence of 100% C6-labelled glucose, again, underlines the critical role of TK and the non-oxidative synthesis of ribose in the tumor proliferation process [9]. The clinical significance of these studies is evidenced by the fact that TK reactions are

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Abbreviations: TK, transketolase; OT, oxythiamine; PC, pentose cycle; DHEA, dehydroepiandrosterone; PI, propidium iodide; DMSO, dimethylsulfoxide; i.p., intraperitoneal; FSC, forward scatter; SSC, side scatter; H, hematoxilin; E, eosin

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currently promoted by the uncontrolled administration of thiamine, the co-factor of this central enzyme reaction in tumor cell proliferation, to cancer patients through total parenteral nutrition, special diets, vitamin supplements and food products [10,11].

Here, we demonstrate that the inhibitory effect of the PC inhibitor drugs, oxythiamine (OT) and dehydroepiandrosterone (DHEA) [12], on tumor cell proliferation is associated with a cell cycle arrest in the G_1 phase. We also demonstrate that this effect of PC inhibitor drugs on the cell cycle is similar to that of deoxyglucose and that on signs of apoptosis, direct cell or host toxicity is present.

2. Materials and methods

2.1. Chemicals

OT, DHEA sulfate, Hoechst 33342, (propidium iodide (PI)), phosphate, citric acid, Dulbecco's phosphate-buffered saline, dimethyl sulfoxide (DMSO) and L-leucyl-methylester were purchased from Sigma. RNAse was purchased from Boehringer and Immuno Floure Mounting Medium was purchased from ICN.

2.2. Animals and tumor cells

Ehrlich's ascites tumor cells were maintained in the abdomen of 12 weeks old 25–30 g C57BL/65 mice and weekly transplanted by intraperitoneal (i.p.) aspiration and inoculation. The animals were housed in a climate-controlled pathogen-limited environment with standard rodent food and water ad libitum. Animals before tumor implantation were selected using a random number chart without regard to treatment group assignment. 20×10^6 tumor cells/mouse (100 μl) were i.p. injected in each experimental animal. Tumor cell viability was checked after each harvesting and before each inoculation by the trypan blue exclusion test. All preparations were highly viable ($>95\%$). Animal studies were conducted according to guidelines accepted by the University Animal Care and Use Committee. Animal experiments were repeated twice, unless specified otherwise in the text.

In vivo drug treatment. 4 Days after tumor implantation when cell growth was ensured by increase of weight, the mice were divided into DHEA-, OT- or combined DHEA-OT-treated and control ($n=6$) groups. A 500 mg/ml solution of DHEA was prepared in 60% DMSO-Dulbecco's phosphate-buffered saline mixture and 500 mg/ml OT solution was prepared in Dulbecco's phosphate-buffered saline and diluted to the required concentrations as in Fig. 1. The separate drug solutions or a combination of both were administrated i.p. for 4 days. Control animals received the maximum concentration of DMSO-Dulbecco's phosphate-buffered saline mixture vehicle for the same length of time as the treated groups. The body weight, food and water intake as well as the physical activity of animals were daily registered. Cells were aspirated and the tumor volume measured using a calibrated syringe. Cell cycle phase distribution, cell viability and apoptosis were measured by flow cytometry and fluorescent microscopy.

2.3. Histology

The animals were killed by dislocation and the liver, kidney and heart were excised, washed and cut into pieces which were fixed in 10% neutral phosphate-buffered formalin, embedded in paraffin. The paraffin blocks were sectioned at 5 microns and stained with H and E for histopathological evaluation. All tissues were evaluated histologically.

2.4. Flow cytometry

2.4.1. Flow cytometry experiments. Flow cytometry experiments were carried out using an Epics ELITE and an Epics XL flow cytometer (Coulter Corporation, Hialeah, FL, USA). Excitation of the tumor cell samples was performed using the 488 nm air-cooled argon ion laser operating at 15 mW. Forward scatter (FSC), side scatter (SSC) and red fluorescence (695 nm) for PI were acquired. Optical alignment was achieved by the optimized signal from 10 nm fluorescent beads using standard procedures recommended by the vendor (Immunocheck, Epics Division). An increased length of time and extended acquisition were used to control the stability of DNA histograms. Aggregates were excluded by gating single cells using their area versus peak fluorescent signal.

2.4.2. Cell cycle phase determination. Fresh tumor cells were diluted in phosphate citrate buffer containing 0.2 M Na_2HPO_4 and 0.1 M citric acid (24:1, pH 7.8). Tumor samples were incubated for 1 h with 5 mM L-leucyl-methylester at 37°C in order to eliminate macrophages from the samples. Cells were stained with 5 μg/ml Hoechst 33342 and analyzed after 30 min at room temperature in the dark. The cell cycle phase distribution profile of tumors was determined by DNA flow histograms of the various cell cycle phases (G_1-G_0, S, G_2-M). Different areas of the DNA histograms were analyzed assuming the Gaussian function of the G_1 and G_2-M peaks and attributing the remaining part of the DNA histogram to cells in the S phase [13,14]. All DNA histograms were collected in three separate loadings and we accepted measurements when the difference was less than 8% between measurements. DNA analysis (ploidy analysis) on single fluorescence histograms was performed using the Multicycle software (Phoenix Flow Systems, San Diego, CA, USA). Cell viability was estimated after 18 μg/ml PI incubation. The argon ion laser adjusted to 15 mW at 488 nm induced sample excitation.

2.4.3. Assessment of apoptosis. Three methods were used in order to check whether OT or DHEA induce apoptosis in Ehrlich's tumor cells of treated mice. As a positive control, vincristine, a common anti-cancer drug which has been described to induce apoptosis [15], was administrated at a single dose of 5 mg/kg [16] to mice hosting Ehrlich ascites tumor cells and in the same conditions.

2.4.3.1. Assessment of apoptosis by flow cytometry. Samples were prepared as described above under determination of cell cycle phases. Hoechst 33342 was excited under 333–364 nm wavelengths, 25 mW and fluorescence was collected using 675 and 395 nm band-pass filters [13–17].

2.4.3.2. Assessment of apoptosis by fluorescent microscopy. Quantification of condensed chromatin was carried out on the same samples used for viability and cell cycle distribution studies which were already stained with PI and Hoechst 3342, respectively. After centrifugation for 7 min at $40\times g$ at room temperature, 12 μl of Immuno Floure Mounting Medium was added to the pellets. Cells were investigated under a Zeiss Axioskop epifluorescence microscope. Pycnotic cells with condensed nuclei and/or shrunken cytoplasm were counted using standard methods.

2.4.3.3. Assessment of apoptosis by a pulse method. Tumor cells were suspended in phosphate citrate buffer and incubated with 5 mM of L-leucyl-methylester during 1 h at 37°C. Cells were stained with 5 μg/ml Hoechst 33342 for 15 s and then analyzed by flow cytometry. Apoptotic cells were identified by their lessened forward light scattering and increased side light scattering [18,19].

2.5. Statistical analysis

Statistical analysis was performed by using the parametric unpaired, two-tailed independent sample t test with 95% confidence intervals ($\mu=2.58$) and $P<0.05$ was considered to indicate significant difference between treated and control groups.

3. Results

3.1. In vivo effect of DHEA and OT treatment on tumor cell growth, the cell cycle and cell death

11 Groups of mice ($n=6$, each) were used to determine the dose dependent effect of DHEA and OT on the growth of the Ehrlich's ascites tumor. Increasing doses from 100 to 500 mg/kg of both DHEA and OT were injected daily during 4 days. The weight of treated tumor bearing mice increased less during the treatment than the weight of the untreated control (control, 33.9–43.86 g; OT, 33.4–35.7 g; DHEA, 32.06–37.2 g). Fig. 1 show that both DHEA and OT decrease both the cell volume and cell number of the tumor comparing to the control. OT inhibited tumor growth by 43% at a dose of 300 mg/kg mice/day. This inhibition was further increased to 84% at a dose of 500 mg/kg mice/day. Whereas DHEA inhibition of tumor growth was 50% at 100 mg/kg mice, a

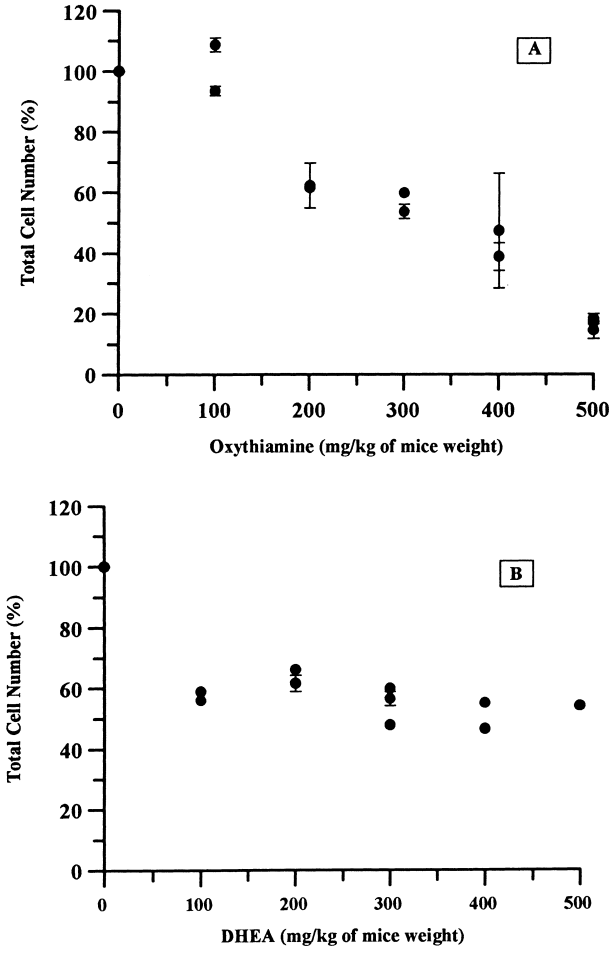

Fig. 1. Dose dependent effect on Ehrlich ascites tumor cell proliferation after 4 days treatment with several doses of A: OT, B: DHEA and combined doses of OT and DHEA. The number of cells after OT, DHEA and combined doses of OT and DHEA treatment are expressed as a percentage of control (100%) cell proliferation (total cell number).

higher DHEA concentration did not further induce any benefic change. Flow cytometry measurement showed that untreated control tumor cells preserved 98% viability which was maintained (over 95%) in tumor cells from all treatments. These results indicated that the inhibition of the PPP through its non-oxidative phase (with OT) is much more effective in decreasing tumor cell proliferation than the inhibition of PPP through its oxidative phase (with DHEA).

To examine the effects of the PPP inhibitors on cell cycle distribution, we subjected fresh tumor cells from mice treated 4 days with 0, 100, 200, 300, 400 and 500 mg/kg of DHEA or OT to flow cytometry analysis of the total DNA content. Fig. 2A and B shows, in percentage of cells, the distribution of cell cycle phases. The percentage of cells in G0-G1 phase increased about 1.5-fold in cells from mice treated with the higher doses of DHEA or OT, whereas the percentage of cells in both S and G2 phases decreased. A dose dependent G1 arrest and S and G2 decrease were observed in the range of 0–400 mg/kg of DHEA or OT. A good correlation exists between this dose dependent G1 arrest and the inhibition of tumor growth (Fig. 1).

Apoptosis was assessed during the flow cytometric cell cycle determination as described by Belloc et al. [13]. Fluorescence emission at 395 and 675 nm in control mice and both OT- and DHEA-treated mice indicates no sign of cell death. Cells from treatment with high doses of these drugs were also analyzed for apoptosis using epifluorescence microscopy. Results from these two methods were in general agreement, showing no apoptosis. These methods were validated using cells from vincristine-treated mice as a positive control. Apoptosis of Ehrlich ascites tumor cells was induced by the administration of a single dose of 5 mg/kg of vincristine to the mice hosts [14]. The laser-beam flow cytometric analysis of tumor cells from vincristine-treated mice displayed a population of cells with decreased fluorescence emission at 395 nm and increased at 695 nm. The combination of both high fluorescence emission at 395 nm and low at 695 nm is an indication of apoptotic cells [18,19]. This population of apoptotic cells was absent in cells from untreated mice.

3.2. *Effects of combined treatment with DHEA and OT on tumor cell growth in vivo*

In order to evaluate the effect of inhibiting simultaneously oxidative (G6PDH) and non-oxidative (TK) pentose phosphate pathways, we have administrated to mice a combination of OT and DHEA at several concentrations. The mixtures of OT and DHEA were injected to groups of six mice each. Since the maximum tumor inhibitory effect was reached at a relatively low dose of DHEA, the dose of DHEA was fixed at 300 mg/kg and OT doses were variable from 200 to 500 mg/kg. A mixture of OT and DHEA at high doses (500 and 400 mg/kg, respectively) was also tested. Table 1 compares the effects of different treatments with OT and DHEA combinations on tumor cell proliferation expressed as percentage growth inhibition. When OT and DHEA were administered separately, for example OT at 400 mg/kg and DHEA at 300 mg/kg, inhibition as measured by the total cell number was reduced by 46.3 and 45.3%, respectively. The reduction or inhibition increased to 86.41% when treatments with drugs at these doses were combined. Similar differences on tumor growth inhibition between separate versus combined treatment with OT and DHEA were observed at other doses of these drugs used. The maximum effect was achieved at the highest concentrations of OT and DHEA administrated simultaneously

Table 1
Growth inhibition effect of combined OT and DHEA treatment at several doses

Number of mixture	OT (mg/kg/day)	DHEA (mg/kg/day)	Growth inhibition (%)
1	300	200	50.2
2	200	300	49.8
3	300	300	69.61
4	400	300	86.4
5	500	300	86.12
6	500	400	94.3

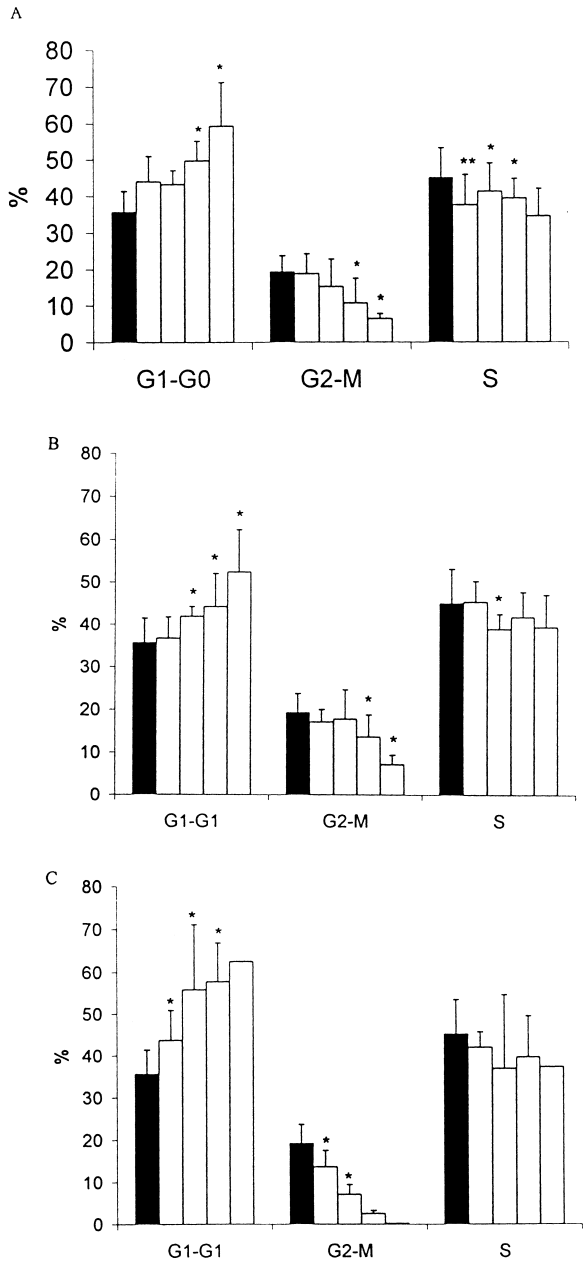

Fig. 2. Cell cycle phase distribution in Ehrlich's tumors after OT treatment. Black bars represent treated control tumor cell cycle frequency distribution (Y axis) as percentage sorting into the G_0-G_1, S and G_2-M phases. Empty bars represent 100, 200, 300 and 400 mg/kg/day drug treatment in the same order. (A) Increasing doses of OT increased cell populations exhibiting G_0-G_1 cycle phases while inhibiting both the S and G_2-M cycles occured after in vivo treatment ($n=6$, values represent mean+S.D., * means significantly different $P<0.05$, $^{**}P<0.01$). (B) Increasing doses of DHEA increased cell populations exhibiting G_0-G_1 cycle phases while inhibiting both the S and G_2-M cycles, although this effect of DHEA was less prominent and not as rapid as after OT treatment ($n=6$, mean+S.D., $^{*}P<0.05$, $^{**}P<0.01$). (C) Empty bars represent 200, 300, 400 and 500 mg/kg/day of OT dose in the same order. The combined treatment resulted in a rapid and significant change in the cell cycle distribution profile, especially affecting the G_2-M phase of tumors and this effect was additive at all doses. The highest dose of combined treatment practically eliminated the G_2-M phase (90.2%). ($n=6$, mean+S.D., $^{*}P<0.05$, $^{**}P<0.01$).

(500 and 400 mg/kg/day for OT and DHEA, respectively). These results show that OT and DHEA are more effective in inhibiting tumor growth when used in combination than used separately and that an inhibition of tumor cell growth of around 94% is achievable at the highest dose of OT and DHEA used. Fig. 2C shows the changes in distribution (in percentage) of cells in different cell cycle phases after different combined DHEA and OT treatments. These results indicate that all mixtures induce an arrest of the cycle at the G1 phase, thus reducing the cell population in the G2 phase comparing to control.

3.3. Direct toxic effect

In order to evaluate any possible direct toxic effect due to the high daily repetitive administration of drugs, we treated eight groups of mice ($n=6$) in the following way. These were first divided into four groups of mice receiving tumor implants and the other four groups serving as healthy control. Mice in each group of the four were either given OT 300 mg/kg, DHEA 200 mg/kg, a mixture of OT 300 and DHEA 200 or vehicle control. Half of the tumor bearing animals were killed and the tumor volume and cell number were registered after 4 days treatment. Since these animals seemed to tolerate these doses of OT and DHEA, we decided to continue the daily injecting for an additional 10 days. The health and weight of the animals were monitored by daily inspections and body weight measurement. All animals were killed after 2 weeks. We found that the percentage of tumor growth inhibition was maintained during the prolonged treatment. The healthy control animals receiving the same doses of drugs showed no change in their behavior or weight after 2 weeks of treatment.

3.4. Histotoxicity

The histotoxicity of DHEA, OT and their mixture was checked on some vital tissue as liver, heart and kidney of the mice hosting Ehrlich's tumor and which were treated with a doses of 300 mg/kg/day DHEA or 400 mg/kg/day OT or a mixture of both. No signs of toxicity were observed in all these tissue as compared to the controls.

4. Discussion

Cell cycle phase distribution determinations by flow cytometry are widely used for the characterization of anti-cancer properties of tumor inhibitory drugs and their effect on cell proliferation. The fundamental task is to measure cell populations with single DNA (G_0-G_1), which are replicated during the S phase followed by the progression towards a completed cell division through the G_2-M phase [18,19]. Oncogenic processes exert their greatest effect by targeting particular regulators of the G_1 phase [20,21]. During preparation in the G_1 phase for DNA replication, cells respond to extracellular signals by either advancing towards a division or withdrawing from the cycle into a resting state (G_0) [22,23]. Malignant tumors are characterized by an increase in their cell populations exhibiting S ($>10\%$) and G_2-M ($>20\%$) phases while cells in their G_0-G_1 ($<70\%$) phases are significantly less frequent than in normal tissue (G_0-$G_1>90\%$, $S<3\%$, G_2-$M<5\%$). Therefore, a G_1 arrest of tumor cells in cancer therapy is a rational and significant goal to achieve.

Flow cytometry is also useful for simultaneous cell cycle analysis and apoptosis detection by FSC and SSC determina-

tions. Our histopathological data indicate no toxic effect of the PC inhibitor drugs on the liver, heart and kidney of the animals treated, while exerting a strong regulatory effect on the cell cycle of the tumor cells consistent with an arrest in the G_1 phase. The health of the animals was not affected by prolonged (2 weeks) treatments with the maximum dose of either drug as indicated by invariable daily food consumption, water intake, daily activity, weight gain and the lack of discomfort or distress.

In the present study, we demonstrated that inhibitors of the oxidative and non-oxidative pentose phosphate pathway reactions regulate the cell division process and induce a G_1 arrest in Ehrlich's ascites tumor cells in mice. Moreover, this effect was dependent on the dose of PC inhibitor drugs given and the two drugs acted synergistically when administered in combination. Both drugs, OT and DHEA, have been described to inhibit cell growth in the Ehrlich ascites tumor model [7] and here, we present new data that these drugs affect tumor cells by causing an arrest in the G_1 phase which was more pronounced after treatment with OT. The strong effect of OT on the regulation of the cell cycle, either alone or in combination with DHEA, has not previously been reported in the medical literature.

One important feature of the resting cell is the limited capacity to synthesize nucleotides. The G_1 arrest observed after treatment with inhibitors of the non-oxidative and oxidative steps of the PC is consistent with their central regulating role in the synthesis of pentose phosphates from glucose and the production of backbone molecules necessary to synthesize nucleic acids de novo or through the salvage pathways of nucleotide bases. The direct inhibition of such a central process is a strong limiting factor for tumor cells to enter the S phase and complete a mitosis. Inhibition of the non-oxidative steps, selectively by OT, is critical for limiting pentose phosphate synthesis since this reaction produces over 70% of the ribose moiety in nucleic acids of cultured or in vivo hosted tumor cells [6,7,9].

Growth factors influence the same pentose cycle pathways, yet, their effect is the opposite to that of PC inhibitors. For example, in vivo administration of the platelet derived growth factor resulted in a significant increase in the levels of PC metabolites in rat liver as early as 5 min after injection [24]. The intracellular levels of PC metabolites, especially that of xylulose-5P which is a central metabolite of the non-oxidative pathway, act as signal molecules in the recruitment of glucose carbons [25] into the cell and regulate cell cycle progression by the expansion of the PC metabolite pool observed in tumor cells [26]. It is very likely that the necessary levels of PC metabolites to enter the S cell cycle phase through 'metabolic priming' are not achieved after treatments with PC inhibitor drugs which induce a withdrawal from the cycle as observed in our studies.

The absence of apoptotic cell death, observed after OT or DHEA treatment directly on tumor cells while strongly regulating the cell cycle, can be explained by the fact that these drugs affect central and specific glucose utilizing metabolic pathways which are involved in nucleic acid synthesis processes while having little effect on glycolysis and intracellular energy production. Tissues exhibiting slow proliferation rates are highly resistant to such treatments due to the low de novo synthesis and high recycling rate of nucleotides.

Benefits from the application of PC inhibitor drugs in the treatment of cancer would also include the 'synchronization' of cancer cells in the G_1 cell cycle phase to increase the efficacy of G_1 specific anti-cancer drugs such as 6-mercaptopurin, thioguanin, prednisolone and tyrphostins [27,28]. PC inhibitors may increase the effect of anti-cancer drugs and allow for a decrease in their toxic doses given in advanced chemotherapy resistant cases of cancer.

In summary, non-oxidative PC reactions offer a new target site for cancer treatment with a strong regulatory effect on the cell cycle. PC inhibitors in a combined treatment offer a new treatment modality in malignancies with emphasis on arresting tumor cell growth instead of killing tumor cells at any expense. This is achieved by taking advantage of the strong subservience of tumor cells on glucose carbons, which are directed toward the synthesis of nucleic acid while other glucose utilizing pathways are not affected.

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