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Effects of ascorbic acid and *DL*-α-tocopherol on human hepatoma cell proliferation and redifferentiation

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KEY WORDS ascorbic acid；vitamin E；hepatocellular carcinoma；cell division；cell differentiation；cultured tumor cells.

ABSTRACT

AIM：To examine the effects of ascorbic acid (AA) and *DL*-α-tocopherol (αT) on the proliferation and redifferentiation of human hepatoma cell. **METHODS**：Choosing an all-trans retinoic acid (RA) as a positive control，cell surface charge，biochemical changes，and cell growth in soft agar were measured. **RESULTS**：After treatment with AA 4 mmol·L^{-1} and αT 1 mmol·L^{-1} together，the growth curve and mitotic index of human hepatoma cells decreased remarkably，the cellular growth inhibitory rate amounted to 61.3 %. The indices related to cell malignancy alleviated significantly；cell surface charge decreased，the electrophoresis rate dropped from 1.64 to 0.89 $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$，the average value of α-fetoprotein (α-FP) content decreased from 300 to 80 $\mu g \cdot g^{-1}$ (protein)，and γ-glutamyl-transpeptidase (γ-GT) activity decreased from 0.81 to 0.201 $U \cdot g^{-1}$ (protein). The index related to cell differentiation increased significantly，such as the average level of tyrosine-α-ketoglutarate transaminase activity increased from 10.6 to 45 $\mu g \cdot g^{-1}$ (protein)，and the colonogenic potential decreased by 96.6 %. **CONCLUSION**：AA and αT combination inhibited human hepatoma cell proliferation，induced redifferentiation，and reversed its malignant phenotypic characteristics.

INTRODUCTION

A number of studies show that malignant tumor cells can be differentiated by some inducers such as retinoic acid (RA)，Me_2SO[1]，superoxide dismutase (SOD)[2]，verbascoside[3]，and ascorbic acid (AA)[4]. Human hepatoma cells have been successfully induced to differentiation in our laboratory[4,5]. Coincidently，most of these inducers，if not all，are effective antioxidants. An increasing amount of experimental and epidemiological evidence implicate the involvement of free radicals in carcinogenesis[6]，and dedifferentiated tumor cells exhibite lower levels of antioxidative activity than their normal，fully differentiated counterparts[2]. Thus，the antioxidants RA，Me_2SO，SOD，verbascoside，and AA may supplement the depletion of antioxidative activity in tumor cells. Hopefully，they may lead to the redifferentiation of malignant tumor cells.

As a strong plasma antioxidant，AA has been found to be able to modulate cell growth and induce cell differentiation in both a leukemia cell line[7] and a solid tumor cell line[4]，and has been used as a leukemia therapeutic agent. The results that AA can induce the differentiation of both leukemia cells and solid tumor cells and the theory that an overload of AA is unlikely to occur in man proved that AA may be used as a general clinical antitumor agent[8]. On the other hand，Podmore *et al*[9] recently found that AA administered as a dietary supplement to healthy humans at a high dose exhibited a prooxidant effect *in vivo*，so it becomes necessary to find a way to treat cancer with a relatively low dose of AA.

AA and α-tocopherol (αT) are respectively water-soluble and lipid-soluble free-radical scavengers，and AA may function synergistically with αT to terminate ROS *in vivo*[10]. A relatively low dose of AA and αT combination may probably offer a general clinically viable method for combating cancer by normalizing tumor cells instead of a high dose of AA.

The differentiation of human hepatoma cells induced by AA and αT combination was examined in this study.

MATERIALS AND METHODS

Reagents Culture media was purchased from

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Gibco Laboratories (Santa Clara, CA). Bovine serum was obtained from Si-Ji-Qing Biotechnology Co (Hangzhou, China). *DL*-α-tocopherol (αT), trypsin, all-trans retinoic acid (RA), Me_2SO were obtained from Sigma (St Louis, MO). All other reagents were of analytical reagent quality.

Cell culture Human hepatoma cell BEL-7402 was set up by Shanghai Institute of Cell Biology, Chinese Academy of Sciences, and cultured in RPMI-1640 medium supplemented with 10 % inactivated bovine serum, benzylpenicillin 100 mU·L^{-1}, streptomycin 100 ng·L^{-1} and $NaHCO_3$ 2.0 g·L^{-1} at 37 ℃. After the cells (8×10^7 cells·L^{-1}) were cultured for 24 h, the culture medium was aspirated and replaced with the culture medium containing AA 4 mmol·L^{-1}, or αT 1 mmol·L^{-1} alone (in Me_2SO 0.02 %), or AA 4 mmol·L^{-1} + αT 1 mmol·L^{-1} (in Me_2SO 0.02 %), or with the medium containing RA 10 μmol·L^{-1} (in Me_2SO 0.02 %) as a positive control. The culture medium was replaced with a fresh medium as the control against AA alone treated group (control 1) or a medium containing Me_2SO 0.02 % as the control against αT, AA + αT or RA treated group (control 2).

Determinations of cell growth curve and mitotic index Cells (8×10^7 cells·L^{-1}) were cultured in 15 mL culture flasks and small bottles with a cover slip strip. The culture flask contained cellular 2 mL suspension and the small bottles contained 1 mL. AA, αT, AA + αT or RA treatments were performed after subculturing for 24 h. The viable cells were counted every day in the first 6 days by trypan blue dye exclusion method. Three small bottles for each group were taken at time. Cells were fixed in Bouin-Hollonde solution and stained with hematoxylin-eosin. The mitotic cells were counted from 1000 cells every day.

Cell electrophoresis The cells treated with AA + αT (4 + 1) mmol·L^{-1} or RA 10 μmol·L^{-1} for 72 h and the control cells were collected and washed with D-Hanks' solution twice, then resuspended at a density of 1 $\times 10^9$ cells·L^{-1}. The cell electrophoresis determination was performed with a round plastic tube electric-bridge filled with NaCl 10 %-agar 1 % and Ag-AgCl electrodes at a direct current voltage 40 V, and room temperature of 24 ℃, taking sucrose 9 % as the electrophoretic medium and using a microcapillary electrophoresis apparatus. The results were expressed by the average time (s) during which a cell moves over a distance of 120 μm and 40 cells in each group were determined. The experiments were repeated three times with similar results. Thus, the result of one experiment will be used as the criterion.

Assays for α-fetoprotein (α-FP) and γ-glutamyltranspeptidase (γ-GT) Two assays were performed at the end of the 72-h treatment. Cell suspension (5×10^8 cells) 0.2 mL was transferred into 0.3 mL of ice-cold lysis buffer containing Tris 5 mmol·L^{-1}, edetic acid 20 mmol·L^{-1} and Triton X-100 0.5 % (vol/vol), pH 8.0, and left to lyse on ice for 30 min before centrifugation at 3000 × *g* for 10 min. The α-FP in the supernatant was determined by α-FP reagent kit (Biological Reagent Research Institute, Lanzhou, China) using ELISA. γ-GT was determined by γ-GT reagent kit (Chemical Reagent Research Institute, Shanghai, China) using azo-coupling reaction. All protein content was measured with the Folin phenol reagent by Lowry's method.

Assay for tyrosine-α-ketoglutarate transaminase (TAT) The TAT activity was detected in the whole cells by the method of Diamondstone[11]. Cell suspension 0.2 mL (in KCl 0.14 mmol·L^{-1}, 5×10^7 cells) was transferred into 0.3 mL of ice-cold lysis buffer described above, and samples were allowed to lyse at 0 ℃, 31 000 × *g* for 30 min. *p*-Hydroxybenzaldehyde 1 μmol produced in the reaction system at 37 ℃ for 30 min was defined as 1 unit of TAT.

Colonogenic assay After 48-h treatment, the treated and untreated cells were washed with RPMI-1640 medium containing 10 % heat-inactivated bovine serum. Cell counts were performed by hemocytometer, and viable cells were assayed by trypan blue exclusion method. An underlayer of 0.5 % agar in RPMI-1640 medium containing 10 % bovine serum was prepared (0.5 mL for each well in a 24-well culture plate). Cells were routinely plated at a concentration of 6×10^6 cells·L^{-1}. Cultures were incubated at 37 ℃ in a 5 % CO_2 humidified air. Cultures were scored 21 d after plating. A colony was defined to be an aggregate of > 50 cells[12].

Statistics Data were expressed as $\bar{x} \pm s$, and compared with *t*-test.

RESULTS

The cell growth rate was slowed down and the doubling time was delayed by AA + αT at 4 + 1 mmol·L^{-1} after subculturing for 24 h, RA also showed similar trend at 10 μmol·L^{-1}, but AA 4 mmol·L^{-1} alone or αT 1 mmol·L^{-1} alone slightly inhibited cell growth compared with the control 1 or 2 (Tab 1).

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Tab 1. Effect of ascorbic acid and α-tocopherol on the proliferation of hepatoma cells for 24 h. $n = 3$ experiments, each experiment contains 3 cultures. $\bar{x} \pm s$. $^aP > 0.05$ *vs* control 1. $^dP > 0.05$, $^fP < 0.01$ *vs* control 2 group (Me_2SO 0.02 %).

	Cell doubling Time/h	10^{-5} × Number of cells		Inhibition /%
		Original	Final	
Control 1	23.6 ± 1.4	2.0	133 ± 12	
AA/4 mmol·L^{-1}	25.5 ± 1.6	2.0	110 ± 10	19.6[a]
Control 2 (Me_2SO 0.02 %)	22.9 ± 1.4	2.0	131 ± 11	
αT/1 mmol·L^{-1}	25.1 ± 1.5	2.0	110 ± 10	20.8[d]
AA + αT/4 + 1 mmol·L^{-1}	33.8 ± 1.7	2.0	51 ± 5	61.3[f]
RA/10 μmol·L^{-1}	31.6 ± 1.8	2.0	64 ± 5	51.8[f]

The inhibitory effect on human hepatoma cell proliferation was increased by AA and αT combination. The survival rate of cells showed no obvious difference (Tab 2).

Tab 2. Effect of ascorbic acid and α-tocopherol treatment for 48 and 72 h on the survival rate of hepatoma cells (Total cells = 1000 in each group). $n = 3$ experiments, each experiment contains 3 cultures. $\bar{x} \pm s$. $^aP > 0.05$ *vs* the control group (Me_2SO 0.02 %).

	Dead cells		Survival rate/%	
	48 h	72 h	48 h	72 h
Control 2	17.0 ± 2.1	17.3 ± 1.9	98.1	98.0
AA + αT/4 + 1 mmol·L^{-1}	50 ± 7	47 ± 5	95.1[a]	94.9[a]
RA/10 μmol·L^{-1}	41 ± 5	42 ± 4	96.2[a]	95.9[a]

The mitotic index shows that hepatoma cells exhibited vigorous proliferating capability with a division peak on the fifth day after subculturing, the mitotic index being 5.21 %. After treatment with RA 10 μmol·L^{-1}, the mitotic index declined to 3.16 %, while after treatment with AA 4 mmol·L^{-1} alone, αT 1 mmol·L^{-1} alone, or AA + αT (4 + 1 mmol·L^{-1}), the mitotic index declined to 4.25 %, 4.15 %, and 2.92 % respectively, and the division peak shifted to the fourth day after subculturing (Fig 1).

After treatment with RA 10 μmol·L^{-1} or AA + αT 4 + 1 mmol·L^{-1} for 6 d, the electrophoresis rate apparently slowed down. The percentage of retardation reached as high as 34.2 % and 43.8 % (Tab 3).

After treatment with RA 10 μmol·L^{-1} or AA + αT 4 + 1 mmol·L^{-1} for 6 d, both the α-FP content and the γ-GT activity decreased markedly, while the average value of TAT activity significantly increased (Tab 4).

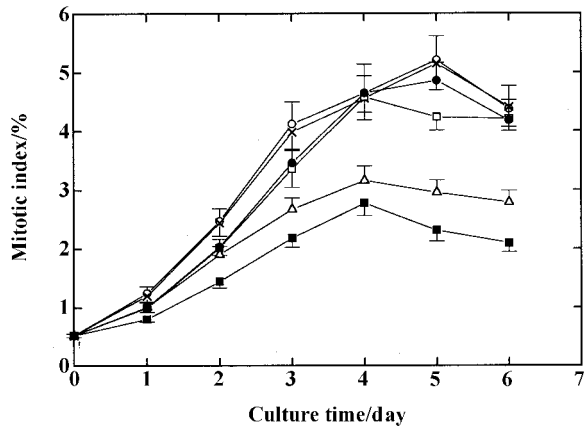

Fig 1. Effect of ascorbic acid and α-tocopherol on the mitotic index of hepatomacytes. Cells were inoculated at a density of 1×10^8 cells·L^{-1}. The cells were cultured in medium with none (○), or AA 4 mmol·L^{-1} (●), or Me_2SO 0.02 % (×), or αT 1 mmol·L^{-1} (□), or AA 4 mmol·L^{-1} + αT 1 mmol·L^{-1} (■), or RA 10 μmol·L^{-1} (△). Means of three parallel experiments were plotted. $n = 3 \times 3$ cultures per condition. $\bar{x} \pm s$.

Tab 3. Effect of ascorbic acid and α-tocopherol on cell surface charge. $n = 3$ experiments, 3 cultures per condition. $\bar{x} \pm s$. $^cP < 0.01$ *vs* the control group.

	Electrophoresis time/s	Electrophoresis rate/μm·s^{-1}·V^{-1}·cm^{-1}	Retardation /%
Control 2	11.18 ± 0.29	1.63	
AA + αT/4 + 1 mmol·L^{-1}	20.0 ± 1.5	0.89	43.8[c]
RA/10 μmol·L^{-1}	17.1 ± 1.5	1.06	34.2[c]

Tab 4. Effects of ascorbic acid and α-tocopherol on α-FP content, γ-GT, and TAT activities of hepatoma cells. $n = 3$ experiments, 3 cultures per condition. $\bar{x} \pm s$. $^cP < 0.01$ *vs* the control group.

	α-FP/μg·g^{-1} (protein)	γ-GT/U·g^{-1} (protein)	TAT/μmol·g^{-1} (protein)
Control 2	300 ± 25	0.81 ± 0.08	10.6 ± 0.9
AA + αT/4 + 1 mmol·L^{-1}	80 ± 6^c	0.201 ± 0.010^c	45 ± 4^c
RA/10 μmol·L^{-1}	112 ± 10^c	0.176 ± 0.016^c	35 ± 3^c

Cells 3000 were plated in 24-well culture plate, and cell colonies of more than 50 cells were counted after 21 d. The colonogenic potential (CP) of treated cells in soft agar decreased significantly (Tab 5).

Tab 5. Effect of ascorbic acid and α-tocopherol on colonogenic potential of hepatomacytes. $n = 3$ experiments, 3 cultures per condition. $\bar{x} \pm s$. $^cP < 0.01$ *vs* the control group.

	Number of colonies	Colonogenic potential/%
Control 2	315 ± 27	100
AA + αT/4 + 1 mmol·L^{-1}	11 ± 3	3.4^c
RA/10 μmol·L^{-1}	34 ± 5	10.6^c

DISCUSSION

Some of the important characteristics of the transformed cells are continuous division and constant multiplication. Therefore, the inhibitory effect on the multiplication of tumor cells is a significant appraisal of induced differentiation. The results in Tab 1, 2 and Fig 1 confirmed that AA and αT combination inhibited the multiplication of hepatoma cells without exhibiting any cytotoxicity.

The net charges on the tumor cell surface are generally more than those in corresponding normal cells, thus, the decrease in the tumor cell electrophoresis rate has been taken as an appraisal of tumor cell differentiation[13]. Our results showed that AA + αT combination decreased the cell surface charge significantly.

The increase in α-FP content and γ-GT activity is related with hepatocytes malignancy, while increase in TAT activity and the decrease in CP are the indices of hepatoma differentiation. Inspecting the CP of cultured cells in soft agar is a very important marker for distinguishing between malignant, benign, or normal cells[5,13]. AA + αT 4 + 1 mmol·L^{-1} treatment was able to increase TAT activity, to decrease the content of α-FP, γ-GT activity, and CP significantly.

Our results showed that with a treatment of AA + αT 4 + 1 mmol·L^{-1}, the malignant characteristics of human hepatoma cells decreased, while the characteristics related with cell normalization increased. All these changes indicated that the hepatoma cells were inclined towards normalization, and confirmed that AA + αT combination possessed the ability of inducing human hepatoma cell redifferentiation and impelling the cells reversion against the malignant phenotype.

ROS may have some important biological roles as intra- and intercellular "messengers" or "signals", promoting tumor cell proliferation and maintaining cell viability[14]. AA and αT are respectively water-soluble and lipid-soluble free-radical scavengers, and AA may function synergistically with αT to terminate ROS[10], so their induction of hepatoma cell differentiation may be related to their antioxidative activity.

A great deal of evidence proves that ROS are related to cell carcinogenesis[6]. This free radical mechanism for carcinogenesis may lead to a new prediction: the tumor cell redifferentiation or normalization may be induced by minimizing the generation of ROS in tumor cells. Our study offers some evidence to support the above.

Normalizing tumor cells, rather than killing them with high cytotoxicity or other side-effects is considered to be a new strategy for combating cancer. Differentiation inducers are expected to be such a new type of antitumor agents. AA successfully induces the differentiation of a leukemia cell line[8] and has been used as a leukemia therapeutic agent. Our previous study shows that AA induced differentiation firstly in solid tumor cells[4]. These results that AA can induce redifferentiation not only in leukemia cells, but also in solid tumor cells prove that AA be used as a general clinical differentiation inducer against cancer. The high dose of AA can not be absorbed completely *in vivo*, and AA administered at a high dose exhibits a prooxidant effect *in vivo*[9,10]. So the results of this study that a relatively low dose of AA and αT combination can induce hepatoma cell redifferentiation may probably offer a more useful and generally viable clinical method for combating cancer. The mechanism of AA and αT combination inducing hepatoma cell redifferentiation is still unclear and needs to be further studied.

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抗坏血酸与 *DL*-α-生育酚对人肝癌细胞增殖与再分化的作用

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关键词 抗坏血酸；维生素 E；肝细胞癌；细胞分裂；细胞分化；培养的肿瘤细胞

目的：测定抗坏血酸(AA)和 *DL*-α-生育酚(αT)联合处理对人肝癌细胞增殖与再分化的作用. **方法**：维甲酸(RA)为阳性对照. 用细胞表面电荷、生化变化和软琼脂细胞生长等指标测定细胞分化和增殖. **结果**：用 AA 4 mmol·L^{-1}和 αT 1 mmol·L^{-1}联合处理后，肝癌细胞的生长和分裂指数显著下降，增殖抑制率达 61.3 %. 与恶化有关的指标显著减轻，如细胞表面电荷明显降低，电泳率从 1.63 μm·s^{-1}·V^{-1}·cm^{-1}降低到 0.89，甲胎蛋白由 300 μg·g^{-1}(protein)降到 80，γ-谷氨酰转肽酶活性由 0.81 U·g^{-1}(protein)降到 0.201. 与分化相关的酪氨酸-α-酮戊二酸转氨酶活性显著上升，由 10.6 μmol·g^{-1}(protein)升高到 45，细胞克隆形成力降低 96.6 %. **结论**：AA 和 αT 合用能够抑制人肝癌细胞增殖，诱导分化，并逆转恶性表型.

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