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Effects of ascorbic acid on human hepatoma cell proliferation and redifferentiation

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KEY WORDS ascorbic acid; tretinoin; hepatocellular carcinoma; cell differentiation; cell division; cell survival; alpha-fetoproteins; mitotic index; electrophoresis; gamma-glutamyltransferase

ABSTRACT

AIM: To examine the effects of ascorbic acid (AA) on hepatoma. **METHODS**: Choosing an all-trans tretinoin (Tre) as a positive control, cell growth, and cell redifferentiation tests by cell surface charges, biochemical changes, and cell growth in soft agar were measured. **RESULTS**: After being treated with AA 6 $mmol \cdot L^{-1}$, the growth curve and mitotic index of human hepatoma cells decreased remarkably, the cellular growth inhibitory rate amounted to 58.9 %. The indices related with cell malignancy alleviated, such as cell surface charge obviously decreased, the electrophoresis rate dropped from 1.64 $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$ to 0.93, the average value of α-fetoprotein (α-FP) content decreased from 302 $\mu g \cdot g^{-1}$ (protein) to 90, and γ-glutamyl-transpeptidase (γ-GT) activity from 0.81 $U \cdot g^{-1}$ (protein) to 0.16. The index related with cell differentiation increased, such as the average level of tyrosine-α-ketoglutarate transaminase activity increased from 10.3 $\mu mol \cdot g^{-1}$ (protein) to 41.2, and the colonogenic potential decreased 94.4 %. **CONCLUSION**: AA can inhibit human hepatoma cells proliferation, induce redifferentiation, and reverse its malignant phenotypic characteristics.

INTRODUCTION

A number of studies showed that malignant tumor cells could be differentiated by some inducers such as tretinoin (Tre), dimethyl sulfoxide (Me_2SO)[1], superoxide dismutase (SOD)[2], and verbascoside[3]. Human hepatoma cells have been a successfully induced to differentiation in our laboratory[4]. Coincidently, most of these inducers, if not all, are effective antioxidants, ie, they can scavenge reactive oxygen species (ROS)[5]. An increasing amount of experimental and epidemiological evidence implicates the involvement of free radicals in carcinogenesis[6], and dedifferentiated tumor cells exhibit lower levels of antioxidative activity than that of their normal, fully differentiated counterparts[7]. Thus, the antioxidants Tre, Me_2SO, SOD, and verbascoside may supplement the depletion of antioxidative activity in tumor cells. Hopefully, they may lead to the redifferentiation of malignant tumor cells.

Ascorbic acid (AA), an essential nutrient in humans is involved in many cellular functions. The epidemiologic evidence and laboratory studies showed that AA was a strong plasma antioxidant that can protect lipids, proteins, and cell membranes from oxidative damage by scavenging oxygen radicals[8]. There had been a considerable discussion on AA and cancer during the last few years. Although some authors believed that AA could protect against cancer, or at least, against some types of cancer, the others seemed to be inclined to doubt such a function for this vitamin. Physiologic mechanisms of AA absorption, tissue uptake, metabolism, and elimination support the theory that an overload of AA is unlikely to occur in man[9]. AA has been found to be able to modulate cell growth and induce cell differentiation in a leukemia cell line[10], and has been used as a leukemia therapeutic agent. So the investigation about whether the AA can induce the differentiation of solid tumor cells may probably offer a general clinical agent for combating cancer by normalizing tumor cells.

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The differentiation of human hepatoma cells induced by AA was examined.

MATERIALS AND METHODS

Reagents Culture media was purchased from Gibco Laboratories (Santa Clara, CA, USA). Bovine serum was obtained from Si-ji-qing Biotechnology Co (Hangzhou, China). Trypsin, all-trans tretinoin (Tre), dimethyl sulfoxide (Me_2SO) were obtained from Sigma (St Louis, MO, USA). All other reagents were of analytical reagent.

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 or 6 mmol · L^{-1} or with the medium containing Tre 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 treated group (control 1) or containing Me_2SO 0.02 % as the control against Tre treated group (control 2).

Determination of cell growth curve and mitotic index Cells of 8×10^7 L^{-1} were cultured in 15 mL culture flasks and small bottles with a cover slip strip. The culture flask contained cellular suspension 2 mL and the small bottles contained 1 mL. AA or Tre treatment was performed after subculturing for 24 h. Three flasks for each of cells from treatment and control groups were collected every day in the first 6 d, the viable cells were counted using the trypan blue dye exclusion method. Three small bottles for each group were taken at the same 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 6 mmol · L^{-1} or Tre 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 1×10^9 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 at 24 ℃, taking sucrose 9 % as the electrophoretic medium and using a microcapillary electrophoresis apparatus. The results are 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 % (V/V), pH 8.0, and left to lyse for 30 min on ice before the centrifugation for 10 min at $3000 \times g$. The α-FP in the supernatant was determined by α-FP reagent kit (Biological Reagent Research Institute, Lanzhou, China) in the way of ELISA bi-antibody with α-FP. 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 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 for 30 min at 0 ℃, $31\,000 \times g$. One μmol *p*-hydroxybenzaldehyde produced in the reaction system at 37 ℃ for 30 min was defined as 1 unit of TAT.

Colonogenic assay After a 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 haemocytometer, and viable cells were assayed by trypan blue exclusion. An under layer of 0.5 % agar in RPMI-1640 medium containing 10 % bovine serum was prepared (0.5 mL in a 24-well culture plate), cells were suspended routinely at a concentration of 6×10^6 cells · L^{-1} in the plating layer 0.5 mL. Cultures were incubated at 37 ℃ in a 5 % CO_2-humidified atmosphere of air. Cultures were scored 21 d after plating. A colony was defined to be an aggregate of

> 50 cells[12].

Statistics Date 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 both at 4 and 6 mmol·L^{-1} after subculturing for 24 h, so did Tre at 10 μmol·L^{-1} (Tab 1).

Tab 1. Effect of AA on the proliferation of hepatomacytes. The numbers given represent the average of triplicate experiments ($\bar{x} \pm s$). $n = 3 \times 3$ cultures per conditions. $^aP > 0.05$, $^cP < 0.01$ vs the corresponding control group.

	Cell doubling time/h	10^{-5} × Number of cells Original	Final	Inhibition /%
AA/mmol·L^{-1}				
0	23.6 ± 1.4	2.0	133 ± 12	
4	25.5 ± 1.6	2.0	110 ± 10	19.6[a]
6	32.8 ± 1.5	2.0	57 ± 5	58.9[c]
Tre/μmol·L^{-1}				
0	22.9 ± 1.4	2.0	131 ± 11	
10	31.6 ± 1.8	2.0	64 ± 5	51.8[c]

The inhibitory effect on human hepatoma cell proliferation was increased by increasing the concentration of AA. And the survival rate of cells showed no obvious difference between the three treated groups and the corresponding control (Tab 2).

The mitotic index showed that hepatoma cells exhibited vigorous proliferating capability with a division peak on the fifth day after subculturing, the mitotic index was 5.21 %. After treatment with Tre 10 μmol·L^{-1}, the mitotic index declined to 3.16 %, while after treatment with AA 4 or 6 mmol·L^{-1}, the mitotic index declined to 4.25 % and 2.89 % respectively, and the division peak shifted to the fourth day after subculturing (Fig 1).

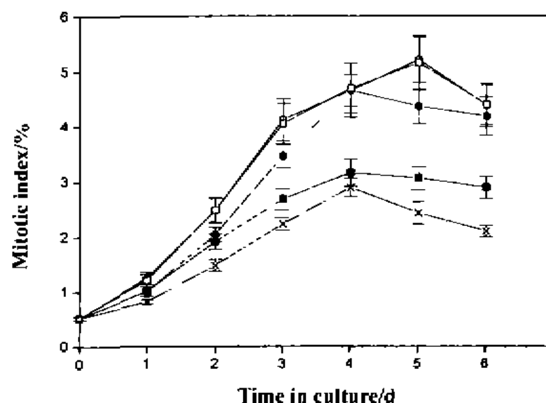

Fig 1. Effect of AA on the mitotic index of hepatomacyte. Cells were inoculated at a density 1×10^8 cells·L^{-1}. The mitotic rate were counted every day. The cells were cultured in medium with none (○), or AA 4 and 6 mmol·L^{-1} (● and ×), or Me_2SO 0.02 % (□), or Tre 10 μmol·L^{-1} (■). Means of three parallel experiments are plotted. $n = 3 \times 3$ cultures per condition.

The results showed in Tab 1, 2, and Fig 1 indicated that AA inhibited hepatoma cells proliferation greatly, and in such a concentration, AA exhibited no cytotoxicity.

After treatment with Tre 10 μmol·L^{-1} or AA 6

Tab 2. Effect of AA treated for 48 and 72 h on the survival rate of hepatomacytes. The numbers given represent the average of triplicate experiments. $n = 3 \times 3$ cultures per conditions. $\bar{x} \pm s$. $^aP > 0.05$ vs the corresponding control group.

	Total cells	Dead cells 48 h	Dead cells 72 h	Survival rate/% 48 h	Survival rate/% 72 h
AA /mmol·L^{-1}					
0	1000	16.6 ± 2.2	17.1 ± 1.9	98.2	98.0
4	1000	15.5 ± 1.9	17.3 ± 1.7	98.6[a]	97.9[a]
6	1000	51.8 ± 6.8	46.8 ± 5.3	95.9[a]	96.1[a]
Tre /μmol·L^{-1}					
0	1000	17.0 ± 2.1	17.3 ± 1.9	98.1	98.0
10	1000	41.3 ± 4.8	42.5 ± 3.9	96.2[a]	95.9[a]

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mmol·L^{-1} for 6 d, the electrophoresis rate apparantly slowed. The percentage of retardation reached as high as 34.2 % and 41.8 % (Tab 3).

Tab 3. Effect of AA on cell surface charge. The experiments were repeated 3 times with similar results, thus taken one experiment as the criterion ($x \pm s$). n=3 cultures per conditions. $^cP<0.01$ vs the corresponding control group.

	Electrophoresis time/s	Electrophoresis rate/ μm·s^{-1}·V^{-1}·cm^{-1}	Retardation/ %
AA/mmol·L^{-1}			
0	11.21±0.34	1.64	
6	19.56±1.54	0.93	41.8^c
Tre/μmol·L^{-1}			
0	11.18±0.29	1.63	
10	17.12±1.47	1.06	34.2^c

After treatment with Tre 10 μmol·L^{-1} or AA 6 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 greatly increased (Tab 4).

Tab 4. Effects of AA on α-FP content, γ-GT and TAT activities of hepatomacytes. The values represent $\bar{x} \pm s$ of experiments performed in triplicate, $n = 3 \times 3$ cultures per conditions. $^cP<0.01$ vs the corresponding control group.

	α-FP/ μg·g^{-1}	γ-GT/ U·g^{-1}	TAT/ μmol·g^{-1}
AA/mmol·L^{-1}			
0	302±24	0.81±0.78	10.3±0.8
6	90±8^c	0.16±0.16^c	41.2±3.6^c
Tre/μmol·L^{-1}			
0	300±25	0.81±0.78	10.6±0.9
10	112±10^c	0.18±0.16^c	34.6±3.2^c

Cells of 3×10^3 were plated in 24-well culture plates, and cells colonies of more than 50 cells were counted after 21 d. The colonogenic potential (CP) of treated cells in soft agar decreased markedly (Tab 5).

Tab 5. Effect of AA on CP of hepatomacyets. The values represent $\bar{x} \pm s$ of experiments performed in triplicate. $n = 3 \times 3$ cultures per condition. $^cP<0.01$ vs the corresponding control group.

	Number of colonies obtained	CP/%
AA/mmol·L^{-1}		
0	320±24	100
6	18±3	5.6^c
Tre/μmol·L^{-1}		
0	315±27	100
10	34±5	10.6^c

DISCUSSION

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 of the proliferation and mitotic index of cells by AA confirm that AA could inhibit the multiplication of hepatoma cells. The survival rate of cells showed no obvious change after treatment with AA or Tre, indicated that the inhibitory effect of AA on hepatoma cells proliferation was not related with their cytotoxicity. Ivanov *et al*[13] also found that AA inhibited significantly the [^{3}H]thymidine incorporation was not due to its cytotoxicity.

The net charges at the tumor cell surface are generally more than those in corresponding normal cells, thus, the cell electrophoresis rate of tumor cells is higher than that of normal cells, and the decrease in the cell electrophoresis rate has been taken as an appraisal of tumor cell differentiation[14]. Our results showed that AA 6 mmol·L^{-1} treatment could decrease the amount of cell surface charge greatly.

The increase of α-FP content and γ-GT activity are the properties related with hepatocytes malignancy, while the increase of TAT activity and the decrease of CP are the indices of hepatoma differentiation. Inspecting the CP of culture cells in soft agar is a very important marker for distinguishing malignant, benign, and normal cells[5,12]. AA 6 mmol·L^{-1} treatment was able to increase TAT activity, to decrease the content of α-FP, γ-GT activity, and CP.

Our results showed that with a treatment of AA 6 mmol·L^{-1}, the malignant characteristics of human hepatomacytes decreased. On the contrary, the characteristics related with cells normalization increased. All these changes indicated that the hepatomacytes were inclined towards normalization, and confirmed that AA possessed the activity of

inducing human hepatoma cells redifferentiation and impelling the tumor cells reversion against the malignant phenotype.

ROS may have some important biological roles in intra- and intercellular "messenger" or "signal", promoting tumor cell proliferation and maintaining cell viability[15]. AA could scavenge ROS effectively[8], so its induction of human hepatoma cells differentiation may be related to its antioxidative activity.

A great deal of evidence proves that ROS relates to cell carcinogenesis[6]. This free radical mechanism for carcinogenesis must lead to a new prediction in the opposite direction: 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 new prediction.

Normalizing tumor cells, rather than by killing tumor cells with high cytotoxicity or other side-effects was considered to be a new strategy for combating cancer. Differentiation inducers are expected to be such a new type of antitumor agent. The epidemiologic evidence and laboratory studies showed that AA was a strong oxygen radicals scavenger[8]. Some authors believed that AA could protect against cancer, or at least, against some types of cancer, and physiological mechanisms of AA absorption, tissue uptake, metabolism, and elimination support that an overload of AA was unlikely to occur in man[9]. AA successfully induces the differentiation of a leukemia cell line[10] and has been used as a leukemia therapeutic agent, while our study induced differentiation first in solid tumor cells by AA. These results that AA can induce the redifferentiation not only in leukemia cells, but also in solid tumor cells proved that AA can be used as a general clinical differentiation inducer against cancer, or at least, against some types of cancer. But its mechanism was still unclear and needed to be further studied.

REFERENCES

1 Barrera G, Pizzimenti S, Muzio G, Maggiora M, Garramone A, Biasi F, *et al*. Enzymatic pattern of adehyde metabolism during HL-60 cell differentiation. Biochem Biophys Res Commun 1996; 223: 73 – 9.

2 St Clair DK, Oberley TD, Muse KE, St Clair WH. Expression of manganese superoxide dismutase promotes cellular differentiation. Free Radic Biol Med 1994; 16: 275 – 82.

3 Li J, Zheng Y, Zhou H, Su BN, Zheng RL. Differentiation of human gastric adenocarcinoma cell line MGc80-3 induced by verbascoside. Planta Med 1997; 63: 499 – 502.

4 Ren JG, Zheng RL, Shi YM, Gong B, Li JF. Apoptosis, redifferentiation and arresting proliferation simultaneously triggered by oxidative stress in human hepatoma cells. Cell Biol Int 1998; 22: 41 – 9.

5 Wang PF, Kang JH, Zheng RL, Yang ZG, Lu JF, Gao JJ, *et al*. Scavenging effects of phenylpropanoid glycosides from *Pedicularis* on superoxide anion and hydroxyl radical by the spin trapping method. Biochem Pharmacol 1996; 51: 687 – 91.

6 Trush MA, Kensler TW. An overview of the relationship between oxidative stress and chemical carcinogenesis. Free Radic Biol Med 1991; 10: 201 – 9.

7 Beckman BS, Balin AK, Allen RG. Superoxide dismutase induces differentiation of Friend erythroleukemia cells. J Cell Physiol 1989; 139: 370 – 6.

8 Frei B, England L, Ames BN. Ascorbate is an outstanding antioxidant in human blood plasma. Proc Natl Acad Sci USA 1989; 86: 6377 – 81.

9 Rivers JM. Safety of high-level vitamin C ingestion. In: Burns JJ, Rivers JM, Machlin LJ, editors. Third conference on vitamin C. New York: The New York Academy of Sciences; 1987. p 445 – 53.

10 Alcain FJ, Buron MI. Ascorbate on cell growth and differentiation. J Bioenerg Biomembr 1994; 26: 393 – 8.

11 Diamondstone TI. Assay of tyrosine transaminase activity by conversion of hydroxybenzaldehyde. Anal Biochem 1966; 16: 395 – 401.

12 San RH, Laspia MF, Soiefer AI, Maslansky CJ, Rice JM, Williams GM. A survey of growth in soft agar and cell surface properties as markers for transformation in adult rat liver epithelial-like cell culture. Cancer Res 1979; 39: 1026 – 34.

13 Ivanov VO, Ivanova SV, Niedzwiecki A. Ascorbate affects proliferation of guinea-pig vascular smooth muscle cells by direct and extracellular matrix mediated effects. J Mol Cell Cardiol 1997; 29: 3293 – 303.

14 Liang ZJ, Shi YD. The technique of cell electrophoresis and its use on biology and medicine. Prog Biochem Biophys (Chinese) 1976; 1: 54 – 62.

15 Khan AU, Wilson T. Reactive oxygen species as cellular messengers. Chem Biol 1995; 2: 437 – 45.

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抗坏血酸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；维甲酸；肝细胞癌；细胞分化；细胞分裂；细胞存活；α-甲胎蛋白类；有丝分裂指数；电泳；γ-谷氨酰转移酶

目的：测定抗坏血酸(AA)对人肝癌细胞增殖与再分化的作用. **方法**：维甲酸(Tre)为阳性对照，测定细胞增殖、细胞表面电荷、生化变化和软琼脂细胞生长等指标. **结果**：用 AA 6 $mmol \cdot L^{-1}$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 58.9 %. 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 1.64 降到 0.93 $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$，甲胎蛋白(α-FP)由 302 $\mu g \cdot g^{-1}$ (protein) 降为 90、γ-谷氨酰转氨酶(γ-GT)活性由 0.81 $U \cdot g^{-1}$(protein)降到 0.16. 酪氨酸-α-酮戊二酸转氨酶(TAT)活性由 10.3 $\mu mol \cdot g^{-1}$(protein)升高为 41.2，细胞克隆形成力降低 94.4 %. **结论**：AA 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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