Institute of Biophysics[1], School of Life Sciences, Lanzhou University, Lanzhou and Yantai University[2], Yantai, People's Republic of China

Ascorbic acid induces redifferentiation and growth inhibition in human hepatoma cells by increasing endogenous hydrogen peroxide

Q. S. ZHENG[1,2], Y. T. ZHANG[1] and R. L. ZHENG[1]

The mechanisms of redifferentiation and growth inhibition induced in human hepatoma cells by ascorbic acid (AA) were studied. After treatment with AA, the content of hydrogen peroxide (H_2O_2) and the activity of superoxide dismutase (SOD) increased in a concentration- and time-dependent manner, while the activity of catalase (CAT) decreased in a concentration- and time-dependent manner. Using 6 mM AA as a positive control, after treatment by 50 μM hydrogen peroxide, the malignant characteristics of human hepatoma cells were alleviated; for example as cell surface charge markedly decreased, the electrophoresis rate dropped from 1.68 $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$ to 0.97, the average of α-fetoprotein content decreased from 327 $\mu g \cdot g^{-1}$ protein to 193, and γ-glutamyl-transpeptidase activity fell from 0.84 $U \cdot g^{-1}$ protein to 0.30. The indexes related to cell differentiation were promoted, such as tyrosine-α-ketoglutarate transaminase activity increased from 17.1 $\mu mol \cdot g^{-1}$ protein to 33.1, and the colonogenic potential decreased by 79.3%. SOD and 3-amino-1,2,4-triazole (AT) exhibited some effects, but there were statistically significant differences between the SOD, AT and H_2O_2 or AA groups. AA induced growth inhibition and redifferentiation of human hepatoma cells through the production of hydrogen peroxide, since addition of SOD (200 units/ml), an enzyme that dismutates superoxide and generates hydrogen peroxide, and AT (1.5 mM), a CAT inhibitor that inhibits the activity of CAT and leads to an increase in H_2O_2 content, showed some inducing changes emphasizing the involvement of reactive oxygen species (ROS) in redifferentiation of hepatoma cells. AA can cause the content of H_2O_2 to increase, and the factor H_2O_2 showed a similar effect to AA on growth and redifferentiation suggests that H_2O_2 is involved in hepatoma cell redifferentiation. In conclusion, these results suggest that AA inhibits tumor growth and induces tumor redifferentiation by virtue of producing H_2O_2.

1. Introduction

The antioxidant properties of ascorbic acid (AA) are well known. Moreover, cytosolic ascorbate plays a significant role in cell defense against the toxic effects of free radicals and reactive oxygen species, although this protective role is not yet fully understood [1]. On the other hand, ascorbate is also able to behave as a prooxidant compound. Thus, an ascorbate-dependent, iron-catalyzed peroxidation has been described [2]. Furthermore, ascorbate accelerates the release of iron from ferritin, stimulating its prooxidant effects [3]. However, there is an intense, open discussion on whether ascorbate can act as a prooxidant under physiological conditions [4–6].

Podmore et al. recently found that AA administered as a dietary supplement to healthy humans exhibits a pro-oxidant, as well as an antioxidant, effect *in vivo* [7]. Lee et al. [8] determined that AA induces lipid hydroperoxide decomposition to the DNA-reactive bifunctional electrophiles 4-oxo-2-nonenal, 4,5-epoxy-2 (*E*)-decenal and 4-hydroxy-2-nonenal. The compound 4,5-epoxy-2 (*E*)-decenal is a precursor of etheno-2′-deoxyadenosine, a highly mutagenic lesion found in human DNA. Ascorbate incubated in buffered solution undergoes autooxidation in the presence of oxygen at 37 °C [9], giving rise to the intermediate free radical (AFR) as the first product of oxidation. AFR behaves both as a one-electron oxidant and as a one-electron reductant [10], explaining both the antioxidative and prooxidant effects described for ascorbate. Although AFR is a relatively stable, non-hazardous biological free radical, ascorbate oxidation seems to contribute to the generation of other free radicals and reactive oxygen species, including hydroxyl or superoxide radical and hydrogen peroxide [2]. The low levels of catalase and peroxidase in cancer cells render them potentially sensitive to ascorbate toxicity [11, 12]. In fact, ascorbic acid has been reported to be cytotoxic for Ehrlich ascites tumor cells [13], and to some human tumors [14], including some leukemia and pediatric tumors [15, 16].

In vitro treatment of prostate cancer cell lines with sodium ascorbate acid (0–10 mM) resulted in a decrease in cell viability and thymidine incorporation into DNA through the production of hydrogen peroxide [17]. In our laboratory, we have successfully induced human hepatoma cells differentiation [18] as well as an increase in H_2O_2 content with 6 mM AA [19]. Our aim in this study is to find out the mechanism for the redifferentiation induced by AA in human hepatoma cells.

2. Investigations, results and discussion

As shown in Fig. 1, AA treatment at different concentrations resulted in a concentration- and time-dependent increase of H_2O_2 content in human hepatoma cells.

After treatment with 2 or 4 mM AA, the content of MDA decreased slightly. But in the 6 or 8 mM AA groups, the content of MDA significantly increased in a time- and concentration-dependent manner. (Fig. 2).

AA treatment resulted in a concentration- and time-dependent decrease of CAT activity of human hepatoma cells (Fig. 3).

After treatment of human hepatoma cells with AA, the activity of SOD increased in a concentration- and time-dependent manner (Fig. 4).

The cell doubling time and proliferation were retarded by AA in a concentration-dependent manner. H_2O_2 as well as SOD or AT also retarded cell doubling time and inhibited cell proliferation (Table 1).

The net charges at the surface of tumor cells are in general more than those in corresponding normal cells, thus, the cell electrophoresis rate of tumor cells should be higher than that of normal cells. Therefore a decrease in the cell electrophoresis rate has been taken as an indicator

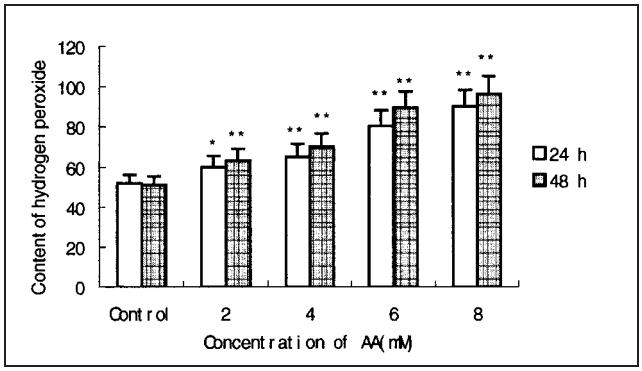

Fig. 1: Effect of ascorbic acid on the content of H_2O_2 in hepatomacytes. Cells were incubated at a density of 1×10^8 cells/ml. The cells were cultured in medium without (control) or with AA 2, 4, 6 or 8 mM for 24 h or 48 h. The content of H_2O_2 is expressed as $\mu mol \cdot mg^{-1}$ protein. Means of three parallel experiments. n = 3 experiments × 3 cultures (mean ± SD). * $P < 0.05$, ** $P < 0.01$ *vs* control group

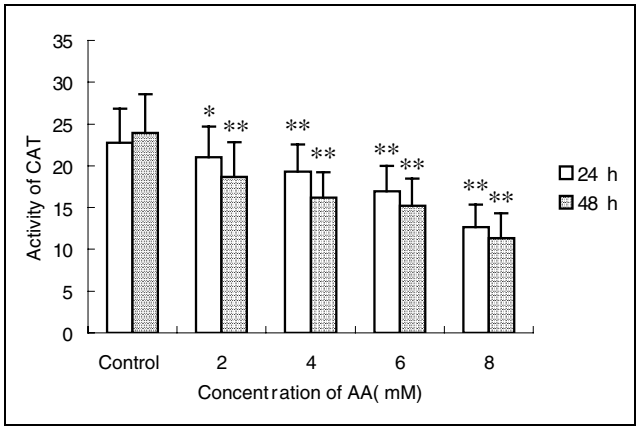

Fig. 3: Effect of ascorbic acid on the activity of CAT in hepatomacytes. Cells were incubated at a density of 1×10^8 cells/ml. The cells were cultured in medium without (control) or with AA 2, 4, 6 or 8 mM for 24 h or 48 h. The activity of CAT is expressed as $U \cdot mg^{-1}$ protein. Means of three parallel experiments. n = 3 experiments × 3 cultures (mean ± SD). * $P < 0.05$, ** $P < 0.01$ *vs* control group

of tumor cell differentiation [20]. After treatment with AA for 6 days, the electrophoresis time was prolonged and the electrophoresis rate was apparently slowed down in a concentration dependent manner. H_2O_2, SOD or AT exhibited similar effects to AA (Table 2).

The increase of the content of α-FP and the activity of γ-GT are related to hepatocyte malignancy, and they have been taken as markers which distinguish malignant and benign hepatomas [21]. After treatment with AA, H_2O_2, SOD or AT, both the α-FP content and the γ-GT activity decreased in a concentration-dependent way (Table 3).

The activity of TAT is higher in normal hepatocytes but reduced during carcinomatous degeneration, and the increase of TAT activity has been regarded as an index related to hepatoma cell differentiation [22]. After treatment with AA, the activity of TAT resulted in a concentration-dependent increase, and the average values of TAT activity significantly increased in the H_2O_2, SOD and AT groups (Table 3).

The colonogenic potential (CP) is a general index of cell differentiation; examining the CP of culture cells in soft agar is a very important marker for distinguishing malignant, benign and normal cells [22, 23]. The colonogenic potential (CP) of cells treated with AA in soft agar decreased significantly in a concentration-dependent manner. H_2O_2, SOD or AT also decreased cell colony formation notably (Table 4).

In our previous studies, we have successfully induced redifferentiation of human hepatoma cells with 6 mM AA [18]. In the present research, we found that with 2, 4, 6 or 8 mM AA treatment, the content of H_2O_2 increased and the activity of CAT decreased, while the activity of SOD increased in time- and concentration-dependent manners; however, the content of MDA was reduced at low concentrations (2 and 4 mM) and was raised at high concentrations (6 and 8 mM). Our interest focuses on the increase of H_2O_2 content and SOD activity and decrease of CAT activity. Choosing AT as a inhibitor of CAT to restrain its activity, no ideal redifferentiation was observed. Promoting the activity of SOD by adding SOD to the medium, the effects of SOD on inducing redifferentiation are insufficient. But, after treatment with H_2O_2, differentiation of human hepatoma cells was induced successfully.

Our results showed that on treatment with AA or H_2O_2 alone, the malignant characteristics of human hepatoma cells were alleviated and the properties related to cell normalization were strengthened. All these changes suggested that the hepatoma cells were inclined towards normaliza-

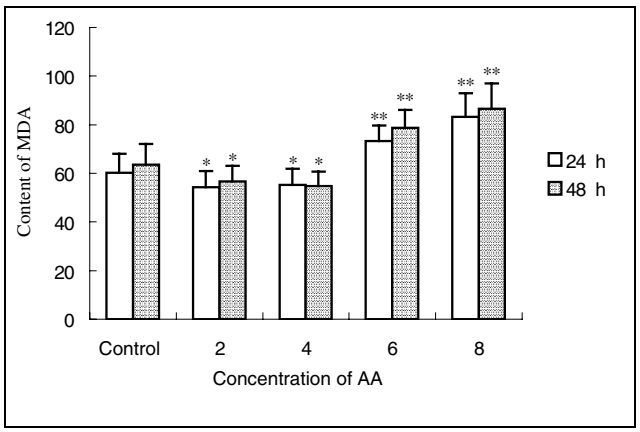

Fig. 2: Effect of ascorbic acid on content of MDA in hepatomacytes. Cells were incubated at a density of 1×10^8 cells/ml. The cells were cultured in medium without (control) or with AA 2, 4, 6 or 8 mM for 24 h or 48 h. The content of MDA is expressed as $ng \cdot mg^{-1}$ protein. Means of three parallel experiments. n = 3 experiments × 3 cultures (mean ± SD). * $P < 0.05$, ** $P < 0.01$ *vs* control group

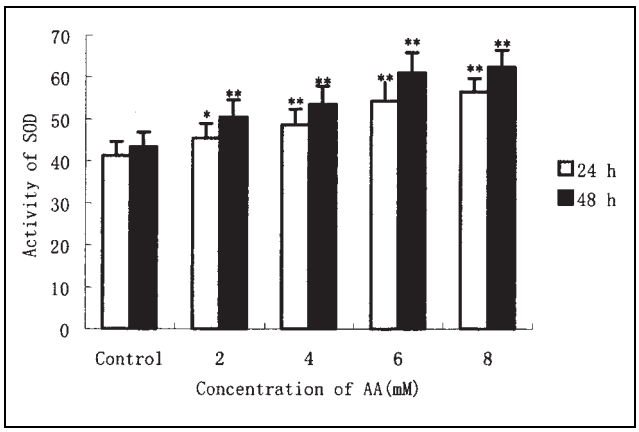

Fig. 4: Effect of ascorbic acid on the activity of SOD in hepatomacytes. Cells were incubated at a density of 1×10^8 cells/ml. The cells were cultured in medium without (control) or with AA 2, 4, 6 or 8 mM for 24 h or 48 h. The activity of SOD is expressed by $U \cdot mg^{-1}$ protein. Means of three parallel experiments. n = 3 experiments × 3 cultures (mean ± SD). * $P < 0.05$, ** $P < 0.01$ *vs* control group

Table 1: Effects of AA, H_2O_2, SOD or AT on human hepatoma cells proliferation

	Cell doubling time (h)	Final cells number ($\times 10^5$)	Inhibition (%)
Control	20.6 ± 2.7	134 ± 21	
AA (2mM)	24.3 ± 2.7	$94 \pm 11^{**}$	29.9
AA (6 mM)	34.5 ± 3.1	$66 \pm 8^{**}$	50.7
AA (8 mM)	38.2 ± 2.9	$57 \pm 8^{**}$	57.5
H_2O_2 (50 μM)	32.7 ± 2.9	$82 \pm 9^{**}$	38.8
SOD (200 U · ml^{-1})	24.8 ± 3.5	$108 \pm 9^{*}$	19.4
AT (1.5 mM)	26.1 ± 2.7	$122 \pm 18^{*}$	9.0

n = 3 experiments × 3 cultures. mean ± SD.
* $P < 0.05$, ** $P < 0.01$ *vs* control group.
The original cells number in each group is 1.8×10^5

tion. The effects of SOD maybe resulted from dismutating superoxide and finally generating H_2O_2, and the effects induced by AT probably occurred through restraining the activity of CAT, finally leading to accumulation of H_2O_2. Therefore, we suggest that H_2O_2 may be implicated in the induction of redifferentiation and the reversion of human hepatoma cells.

The elevation of H_2O_2 induced by AA may be influenced by enhancing SOD activity and inhibiting CAT activity. The effects of AA on the activities of SOD and CAT and lipid peroxide levels in chicken erythrocytes have been investigated. SOD activity in AA groups was increased by 20%, while, CAT activities in the liver and heart of the AA group were significantly decreased by 32% [25]. Another study has shown that the induction of SOD activity can lead to cell differentiation [26]. All these are consistent with our results.

Another study described the effects of AA treatment of two androgen independent prostate cancer cell lines from human (PC3) and rat (Mat-Ly-Lu or MLL) sources. Treatment of PC3 and MLL with sodium ascorbate acid (0–10 mM) resulted in a decrease in cell viability and thymidine incorporation into DNA. These effects of AA were concentration and time dependent. Ascorbate induced these changes through the production of hydrogen peroxide [17].

Autoxidation of ascorbic acid or thiols present with the guanylate cyclase preparation leads to generation of H_2O_2, and its metabolism by bovine liver catalase mediates the concomitant activation of guanylate cyclase [24].

Simultaneous addition of AA and organic hydroperoxides to rat liver microsomes resulted in enhanced lipid peroxidation (approximately threefold) relative to incubation of organic hydroperoxides with microsomes alone. The stimulatory effect of ascorbate on linoleic acid hydroperoxide (LAHP)-dependent peroxidation was evident at all times whereas stimulation of cumene hydroperoxide (CHP)-dependent peroxidation occurred after a lag phase

Table 2: Effects of AA, H_2O_2, SOD or AT on the cell electrophoresis rate

	Electrophoresis Time (s)	Electrophoresis Rate (μm · s^{-1} · V^{-1} · cm^{-1})	Retardation (%)
Control	9.4 ± 1.7	1.68 ± 0.24	
AA (2 mM)	12.5 ± 1.7	$1.35 \pm 0.21^{*}$	19.6
AA (6 mM)	17.8 ± 2.4	$1.09 \pm 0.18^{**}$	35.1
AA (8 mM)	19.4 ± 2.3	$0.93 \pm 0.17^{**}$	44.6
H_2O_2 (50 μM)	19.7 ± 2.1	$0.97 \pm 0.21^{**}$	42.3
SOD (200 U · ml^{-1})	14.9 ± 1.3	$1.39 \pm 0.16^{*}$	17.3
AT (1.5 mM)	13.7 ± 1.5	$1.44 \pm 0.26^{*}$	14.3

n = 3 experiments × 3 cultures. mean ± SD.
* $P < 0.05$, ** $P < 0.01$ *vs* control group

Table 3: Effects of AA, H_2O_2, SOD or AT on the α-FP content, γ-GT and TAT activities of hepatoma cells

	α-FP (μg · g^{-1} protein)	γ-GT (U · g^{-1} protein)	TAT (μmol · g^{-1}protein)
Control	327 ± 22	0.84 ± 0.16	17.1 ± 1.4
AA (2 mM)	$287 \pm 19^{*}$	$0.58 \pm 0.14^{**}$	$23.5 \pm 1.9^{**}$
AA (6 mM)	$169 \pm 15^{**}$	$0.27 \pm 0.07^{**}$	$36.2 \pm 2.5^{**}$
AA (8 mM)	$157+16^{**}$	$0.24 \pm 0.07^{**}$	$44.3 \pm 2.7^{**}$
H_2O_2 (50 μM)	$193 \pm 18^{**}$	$0.30 \pm 0.09^{**}$	$33.1 \pm 2.1^{**}$
SOD (200 U · ml^{-1})	$267 \pm 16^{**}$	$0.61 \pm 0.07^{*}$	$25.4 \pm 2.3^{**}$
AT (1.5 mM)	$291 \pm 23^{*}$	$0.72 \pm 0.15^{*}$	$19.8 \pm 1.4^{*}$

n = 3 experiments × 3 cultures. mean ± SD.
* $P < 0.05$, ** $P < 0.01$ *vs* control group

of up to 20 min. EDTA did not inhibit CHP-dependent lipid peroxidation but completely abolished ascorbate enhancement of lipid peroxidation. Likewise, EDTA did not significantly inhibit peroxidation by LAHP but dramatically reduced ascorbate enhancement of lipid peroxidation. The results reveal a synergistic prooxidant effect of AA on hydroperoxide-dependent lipid peroxidation. The inhibitory effect of EDTA on enhanced peroxidation suggests a possible role for endogenous metals mobilized by hydroperoxide-dependent oxidations of microsomal components [27]. Lee et al. recently found that AA generates bifunctional electrophiles explaining why hydroperoxide-dependent lipid peroxidation is enhanced by AA *in vitro* [8].

Addition of reducing agents to commonly used cell-culture media can lead to generation of substantial amounts of H_2O_2. Some or all of the reported effects of AA and polyphenolic compounds (e.g., quercetin, catechin, epigallocatechin, epigallocatechin gallate) on cells in culture may be due to H_2O_2 generation by interaction of these compounds with cell culture media [28].

Some authors report inhibition of cell death by ascorbate, whereas others demonstrate that ascorbate is cytotoxic. The toxicity of ascorbate is due to ascorbate-mediated production of H_2O_2, to an extent that varies with the medium used to culture the cells. For example, 1 mM ascorbate generates 161 mM H_2O_2 in Dulbecco's modified Eagle's medium and induces apoptosis in 50% of HL60 cells, whereas in RPMI 1640 only 83 mM H_2O_2 is produced, and no apotosis was induced. Direct addition of H_2O_2 at the above concentrations to the cells has similar effects to ascorbate. The ability of ascorbate to interact with different cell culture media to produce H_2O_2 at different rates could account for many or all of the conflicting results obtained using ascorbate in cultured cell assays [29].

Hydrogen peroxide is widely regarded as a cytotoxic agent whose levels must be minimized by the action of antioxidant defence enzymes. Levels of H_2O_2 in the human body may be controlled not only by catabolism but

Table 4: Effects of AA, H_2O_2, SOD or AT on colonogenic potential of hepatoma cells

	Number of colonies	Colonogenic potential (%)
Control	305 ± 27	100
AA (2 mM)	$201 \pm 16^{**}$	65.9
AA (6 mM)	$78 \pm 8^{**}$	25.6
AA (8 mM)	$62 \pm 5^{**}$	20.3
H_2O_2 (50 μM)	$63 \pm 5^{**}$	20.7
SOD (200 U · ml^{-1})	$172 \pm 8^{**}$	56.4
AT (1.5 mM)	$264 \pm 25^{**}$	86.6

n = 3 experiments × 3 cultures. mean ± SD.
** $P < 0.01$ *vs* control group

also by excretion, and H_2O_2 could play a role in the regulation of renal function and as an antibacterial agent in the urine. Recently research has revealed that H_2O_2 has an important role in signal transduction [30] and regulation of gene expression [31].

Although the prooxidant role of ascorbic acid cannot be neglected, under normal physiological conditions ascorbate mainly behaves as a first-order antioxidant that protects cellular components from free radical-induced damage by direct quenching of soluble free radicals or by scavenging those radicals that can initiate lipid peroxidation [32].

Our results suggest that AA may be a potent anticancer agent for human hepatoma cells.

3. Experimental

3.1. Reagents

RPMI-1640 was purchased from Gibco Laboratories (Santa Clara,CA). Bovine serum was obtained from Si-Ji-Qing Biotechnology Co(Hangzhou, China). Trypsin was obtained from Sigma (St Louis, MO, USA). All other reagents are analytical purity.

3.2. Cell culture

Human hepatoma cell BEL-7402 was set up by Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cells were grown in RPMI-1640 medium containing 10% inactivated bovine serum, streptomycin (100 μg/ml), penicillin (100 units/ml), and $NaHCO_3$ 2.0 g/l, and were maintained at 37 °C in a humidified atmosphere of 5% CO_2. After the cells at 10^8/ml were cultured for 24 h, the culture medium was aspirated and replaced with the culture medium containing 2, 4, 6 or 8 mM AA. After adding AA to the medium, pH was adjusted to 7.1 with 1 N NaOH.

3.3. Assays for activities of superoxide dismutase (SOD) and catalase (CAT)

Cellular lysates were prepared by sonication of scraped cells in phosphate-buffered saline (PBS), pH 7.4, containing 0.05% deoxycholate. An aliquot of cell lysate was used for protein estimation. CAT activity was measured by the method of Aebi [33] with some modification [34]. SOD activity was measured according to Sun and Zigman [35]. Protein content was measured with the Folin phenol reagent by Lowry's method, with BSA as a standard.

3.4. Assays for the content of malondialdehyde (MDA) and H_2O_2

Cell lipid peroxides were determined by measuring MDA formation according to Wong et al. [36]. H_2O_2 in cells was determined fluorometrically on 0.15 ml aliquots of the supernatant, using 25 μg p-hydroxyphenyl acetate (PHPA) and 80 μg horseradish peroxidase (HRP) [37] with the excitation and emission wavelengths of 300 and 420 nm. The nonfluorescent substrate PHPA was oxidized to the stable fluorescent product, 2,2′-dihydroxybiphenyl-5,5′-diacetate [$(PHPA)_2$], via the enzymatic reduction of H_2O_2 by horseradish peroxidase (HRP).

3.5. Determination of cell doubling time

The viable cells were counted every day for the first 8 days by the trypan blue dye exclusion method. The cell doubling time is calculated as following: $T_D = 0.693\ (T_2 - T_1)\ /\ \ln\ (N_2/N_1)$, T_D is the cell doubling time from T_1 to T_2, N_1 and N_2 is the cell number at T_1 and T_2 [38].

3.6. Cell electrophoresis

The cells were collected and washed with D-Hanks' solution twice, then resuspended at a density of 1×10^9 cells/l. The cell electrophoresis determination was performed with a round plastic tube electrical-bridge filled with NaCl 10%−agar 1% and Ag−AgCl electrodes at a direct current voltage 40 V, room temperature of 24 °C, using sucrose 9% as the electrophoretic medium. The results were expressed as the average time for a cell to move 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.

3.7. Assays for α-fetoprotein (α-FP) and γ-glutamyl-transpeptidase (γ-GT) cell suspension

0.2 ml (5×10^8 cells) was transferred into 0.3 ml of ice-cold lysis buffer containing Tris 5 mM, edetic acid 20 mM and Triton X-100 0.5% (vol/vol), pH 8.0, and left to lyse on ice for 30 min before centrifugation at $3000\times g$ for 10 min. The α-FP in the supernatant was determined with an α-FP reagent kit (Biological Produce Research Institute, Lanzhou, China) using ELISA. γ-GT was determined with an γ-GT reagent kit (Chemical Reagent Research Institute, Lanzhou, China). Protein content was measured with the Folin phenol reagent by Lowry's method.

3.8. Assay for tyrosine-α-ketoglutarate transaminase (TAT)

The TAT activity in whole cells was detected by the method of Diamondstone (39). Cell suspension 0.2 ml (in KCl 0.14 mM, 1×10^8 cells/ml) was transferred into 0.3 ml of ice-cold lysis buffer as described above, and samples were allowed to lyse at 0 °C, $31000\times g$ for 30 min. *p*-Hydroxybenzaldehyde 1 μmol produced in the reaction system at 30 °C for 30 min was defined as 1 unit of TAT.

3.9. Colonogenic assay

After 48-h treatment, 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 the trypan blue exclusion method. The cells were plated in 24-well culture plates in a double layer nutrient agar system (40). The medium was RPMI-1640 with a final concentration of 10% bovine serum. Cells were routinely plated at a concentration of 6×10^6 cells/ml (0.5 ml for each well in a 24-well culture plate). The plates were incubated for 21 days at 37 °C in a humidified air of 5% CO_2. A colony was defined to be an aggregate of >50 cells [41].

References

1 Antonenkov, V. D.; Sies, H.: Biol Chem Hoppe Seyler. **373**, 111 (1992)
2 Miller, D. M.; Aust, S. D.: Arch. Biochem. Biophys. **271**, 113 (1989)
3 Boyer, R. F.; McCleary, C. J.: Free Radic. Biol. Med. **3**, 389 (1987)
4 Levine, M.; Daruwala, R. C.; Park, J. B.; Rumsey, S. C.; Wang, Y.: Nature **395**, 231 (1998)
5 Poulsen, H. E.; Wiemann, A.; Salonen, J. T.; Nyyssönen, K.; Loft, S.; Cadet, J.; Douki, T.; Ravanat, J. L.: Nature **395**, 232 (1998)
6 Podmore, I. D.; Griffiths, H. R.; Herbert, K. E.; Mistry, N.; Mistry, P.; Lunec, J.: Nature **395**, 232 (1998)
7 Podmore, I. D.; Griffiths, H. R; Herbert K. E; Mistry N.; Mistry, P.; Lunec J.: Nature **392**, 559 (1998)
8 Lee, S. H.; Tomoyuki, O. I.; Blair, I. A.: Science **292**, 2083 (2001)
9 Winkler, B. S.: Biochim. Biophys. Acta **925**, 258 (1987)
10 Coassin, M.; Tomasi, A.; Vannini, V.; Ursini, F.: Arch. Biochem. Biophys. **290**, 458 (1991)
11 Park, C. H.; Amare, M.; Savin, M. A.; Hoogstraten, B.: Cancer Res. **40**, 1062 (1980)
12 Prasad, K. N.; Sinha, P. K.; Ramanujam, M.; Sakamoto, A.: Proc. Natl. Acad. Sci. USA. **76**, 829 (1979)
13 Benade, L.; Howard, T.; Burk, D.: Oncology **23**, 33 (1969)
14 Cameron, E.; Pauling, L.; Leibovitz, B.: Cancer Res. **39**, 663 (1979)
15 Pavelic, K.: Res. **342**, 369 (1985)
16 Medina, M. A.; Garcia de Veas, R.; Schweigerer, L.: Biochem. Mol. Biol. Int. **34**, 871 (1994)
17 Menon, M.; Maramag ,C.; Malhotra, R. K.; Seethalakshmi, L.: Cancer Biochem. Biophys. **16**, 17 (1998)
18 Kang, J. H.; Shi, Y. M.; Zheng, R. L.: **11,** 1019 (1999)
19 Zheng, Q. S.; Zheng, R. L.: Pharmazie **57**, 265 (2002)
20 Liang, Z. J. ; Shi, Y. D.: Prog. Biochem. Biophys. (Chinese) **54**, 62 (1976)
21 Hong, J. T.; Wilson, M. W.; Glauert, H. P.: J. Biochem. Toxicol. **5,** 239 (1995)
22 Yeoh, G. C.; Bennett, F. A.; Oliver, I. T.: Biochem. J. **180,** 153 (1979)
23 Ohashi, E.; Hong, S. H.; Takahashi, T.; Nakagawa, T.; Mochizuki, M.; Nishimura, R; Sasak, N.: J. Vet. Med. Sci. **1**, 83 (2001)
24 Ji, L.; Dillon, L. D.; Wu, E.: Am. J. Physiol. **258** (*Regulatory Integrative Comp. Physiol*. 27): R918-R923, (1990)
25 Sun, M.; Zigman, S.: Anal. Biochem. **90**, 81 (1978)
26 Aebi, H.: Methods Enzymol. **105**, 121 (1984)
27 Wong, S. H. Y.; Knight, J. A.; Hopfer, S. M.; Zaharia, O.; Leach, C. N.; Sunderman, F. W. J.: Clin. Chem. **33**, 214 (1987)
28 Schraufstatter, I. U.; Hinshaw, D. B.; Hyslop, P. A.; Sprang, R. G.; Cochrane, C. G.: J. Clin. Invest. **76**, 1131 (1985)
29 Gadbois, D. M.; Crissman, H. A.; Nastasi, A.; Habbersett, R.; Wang, S. K.; Chen, D.; Lehnert, B. E.: Radiat. Res. **4,** 414 (1996)
30 Diamondstone, T. I.: Anal. Biochem. **16**, 395 (1966)
31 Ng K. W.; Livesey, S. A.; Collier, F.; Gummer, P. R.; Martin, T. J.: Cancer Res. **45**, 5106 (1985)
32 San, R. H. C.; Laspia, M. F.; Soiefer, A. I.; Maslansky, C. J.; Rice, J. M.; Williams, G. M.: Cancer Res. **39**, 1026 (1979)
33 Cherry, P. D.; Wolin, M. S.: Free Radic. Biol. Med. **5,** 485 (1989)
34 Ozturk-Urek, R.; Bozkaya, L. A.; Tarhan, L.: Cell Biochem. Funct. **2**, 125 (2001)

35 Smith, L. E.; Sweet, E.; Freedman, S.; D'Amore, P. A.: Invest. Ophthalmol. Vis. Sci. **33**, 36 (1992)
36 Laudicina, D. C.; Marnett, L. J.: Arch. Biochem. Biophys. **278**, 73 (1990)
37 Halliwell, B.; Clement, M. V.; Ramalingam, J.; Long, L. H.: IUBMB Life **50,** 251 (2000)
38 Clement, M. V.; Ramalingam, J.; Long, L. H.; Halliwell, B.: Antioxid. Redox Signal. **3,** 157 (2001)
39 Sano, M.; Fukuda, K.; Sato, T.; Kawaguchi, H.; Suematsu, M.; Matsuda, S.; Koyasu, S.; Matsui, H.; Amauchi-Takihara, K.; Harada, M.; Saito, Y.; Ogawa, S.: Circ. Res. **89,** 661 (2001)
40 Mendoza, L.; Carrascal, T.; De Luca, M.; Fuentes, A. M.; Salado, C.; Blanco, J.; Vidal-Vanaclocha, F.: Hepatology **34,** 298 (2001)
41 Carr, A.; Frei, B.: FASEB J. **13,** 1007 (1999)

Received February 14, 2002
Accepted March 18, 2002

Prof. Rong-liang Zheng
Institute of Biophysics
School of Life Sciences
Lanzhou University
Lanzhou, Gansu 730000
P.R. China
zhengrl@lzu.edu.cn