

1'-Acetoxychavicol acetate-induced cytotoxicity is accompanied by a rapid and drastic modulation of glutathione metabolism

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Abstract The effect of 1'-acetoxychavicol acetate (ACA), an anticarcinogenic compound naturally obtained from rhizomes and seeds of South East Asia plants, on the intracellular concentration of glutathione and the activities of enzymes related to glutathione metabolism was studied in Ehrlich ascites tumor cells. We showed in a previous study that ACA induced apoptosis in tumor cells and the cell death was reversed by the addition of *N*-acetylcysteine or glutathione ethylester. Here we found that ACA caused a rapid decrease in glutathione level in less than 10 min after ACA exposure. At the time, glutathione reductase activity was significantly inhibited and gamma-glutamyl cysteine increased by ACA exposure. These results show that ACA caused the decrease in the intracellular GSH levels in Ehrlich ascites tumor cells, suggesting that ACA-induced decrease of the cellular GSH levels can lead to growth arrest of cancer and enhancement of the efficacy other anticancer drugs.

Keywords 1'-Acetoxychavicol acetate · Ehrlich ascites tumor cells · Glutathione · Glutathione reductase · Glutathione peroxidase · Polyamines

Introduction

A broad-spectrum anticarcinogenic activity of 1'-acetoxychavicol acetate (ACA), a compound naturally obtained from rhizomes and seeds of South East Asia plants, has been demonstrated in a number of in vivo studies. ACA exhibited chemopreventive effects on chemically induced tumor formation in mouse skin (Murakami et al. 1996), rat oral (Ohnishi et al. 1996), colon (Tanaka et al. 1997a, b), esophagus (Kawabata et al. 2000) and pancreatic (Miyauchi et al. 2000) tumor formation. In addition to these in vivo observations, ACA was able to induce apoptosis in Ehrlich ascites tumor cells in vitro through modulation of polyamine metabolism and caspase-3 activation (Moffatt et al. 2000). We also have shown that protein phosphorylation and reduction of intracellular sulfhydryl groups are involved in the death of Ehrlich ascites tumor cells subjected to ACA treatment (Moffatt et al. 2002). These findings fairly represent mechanisms by which ACA exerts its chemopreventive role. Glutathione is the most abundant antioxidant in the cell, where it is formed predominantly in two redox forms; reduced (GSH) and oxidized (GSSG). Its protective action is based on oxidation of the thiol group of its cysteine residue with the formation of GSSG (Meister and Anderson 1983). The level of reduced GSH is maintained by a functional glutathione reductase or its synthesis via the rate-limiting enzyme gamma-glutamylcysteine synthetase and GSH synthetase. Upon oxidative stress, GSSG may either recycle to GSH or exit from the cells, leading to overall glutathione depletion (Reed 1990). Beside its function as intracellular redox buffer, GSH plays multiple roles in cell physiology, detoxification of toxic chemicals and electrophiles, and regulation of cell death. Several evidences from the last few years also support the idea that GSH decrease is often associated with the apoptotic program. We demonstrated

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that a green tea galloyl polyphenols, epigallocatechin, caused a dose-dependent accumulation of cells in the G0/sub-G1 phase and a related decrease in the phosphorylation of retinoblastoma protein in a cellular thiol-dependent manner, in its growth inhibitory effect in Ehrlich ascites tumor cells (Kennedy et al. 2002). Ghibelli et al. (1995, 1998, 1999) also demonstrated the fundamental role that GSH plays in commitment of programmed cell death. In this study, we examined the intracellular concentration of GSH and GSSG and the activity of glutathione reductase (GR), a critical enzyme for the maintenance of the cellular GSH pool and other enzymes related to glutathione metabolism after ACA exposure in Ehrlich ascites tumor cells.

Materials and methods

Chemicals

ACA was isolated from *Languas galanga* as previously reported (Murakami et al. 1996). Glutathione ethyl ester (GEE), digitonin, 1-chloro-2,4-dinitrobenzene (CDNB), glutathione reductase (GR) and L-gamma-glutamyl-L-cysteine (γ -EC) were obtained from Sigma. *t*-Butyl hydroperoxide was obtained from Aldrich Chemical Company. Tris(2-carboxyethyl)-phosphate (TCEP) was obtained from Pierce (Rockford, IL, USA). Fetal calf serum (FCS) was purchased from JRH Bioscience (Lenexa, Australia). Glutathione (GSH), β -nicotinamide adenine dinucleotide phosphate reduced form (NADPH), *N*-acetyl-L-cysteine (NAC), glutathione disulfide (GSSG), o-phthalaldehyde (OPA), ethylenediamine-N,N,N',N' tetraacetic acid dihydrate (EDTA), trypan blue and 2-amino-2-hydroxymethyl 1,3-propanediol (Tris) were purchased from Wako (Osaka, Japan). All other chemicals used were of commercial grade.

Cell culture

Ehrlich ascites tumor cells were cultured in a humidified atmosphere of 5% CO₂ and 95% air at 37°C for 3–4 days in Eagle's minimum essential medium containing 10% FCS, washed and cultured again at a concentration of 1×10^6 cells/ml in fresh medium. ACA was dissolved in dimethyl sulfoxide (DMSO) and diluted in cultured medium immediately before use (final DMSO concentration <0.5%). In all experiments control cells were incubated in medium and DMSO only.

Assay of cell viability

Cell viability was determined by Trypan blue exclusion assay. Briefly, cells (1×10^6 cells/ml) treated with

DMSO or 40 μ M ACA or 40 μ M ACA with 1 mM GEE or 3 mM NAC were incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37°C in Eagle's minimum essential medium containing 10% FCS for 24 h. To a cell suspension was added an equal volume of 0.4% Trypan blue and the number of viable cells was evaluated under a field microscope. Assays were performed in triplicate.

Determination of intracellular GSH, gamma-EC and GSSG by HPLC

Intracellular GSH, gamma-EC and GSSG was determined according to the method of Sack et al. (2000). Cells were collected by centrifugation and extracted with Tris-HCl buffer (25 mM Tris adjusted to pH 7.5 with HCl) by freeze and thawing. The supernatant was used for HPLC assay. For the assay of GSH and gamma-EC, 480 μ l of supernatant was mixed with 500 μ l of borate buffer (0.56 N boric acid adjusted to pH 10.4 with NaOH) and 73 μ l of OPA solution (10 mg/ml OPA in 10% methanol). Gamma-EC and GSH were separated on an ODS-II column (4.6 $\times$ 150 mm, particle size 5 μ m, Shimadzu Techno-Research, Kyoto Japan) using solvent A (30 mM Sodium acetate adjusted to pH 6.0 with acetic acid) and B (92.3% methanol/7.7% acetonitrile, v/v). Sample was eluted with 96% solvent A and 4% solvent B, and then with a programmed solvent gradient using a linear gradient curve. The gradient changed from 4 to 10% solvent B from 0 to 5 min, from 10 to 14.9% solvent B from 5 to 15 min, from 14.9 to 4% solvent B from 20 to 21 min at a flow rate of 0.45 ml/min. The fluorescence of eluted fractions was measured at excitation and emission wavelength of 230 and 445 nm, respectively, for assay of GSH and gamma-EC with an RF1520 fluorescence monitor (Jasco, Japan). The protein content of the pellet was assayed according to the Bradford (1976) using bovine serum albumin as a reference.

For the assay of GSSG, 35 μ l of 25 mM TCEP was added to 315 μ l of the supernatant. GSSG was then reduced and changed to GSH. After addition of 365 μ l of borate buffer and 53 μ l of OPA solution, total GSH was determined by HPLC as described earlier. Concentration of GSSG was deduced as: $2[\text{GSSG}] = [\text{total GSH}] - [\text{GSH}]$.

Assay of glutathione reductase (GR) activity

GR activity was measured according to the method of Carlberg and Manneervik (1985). Approximately 2×10^6 cells were plated in 60 mm dish and incubated with DMSO

or 40 μM ACA for 0–120 min. After incubation, cells were collected by centrifugation and extracted with 100 μl /dish of 50 mM of potassium phosphate buffer (containing 1 mM EDTA and 0.25 M sucrose adjusted to pH 7.0) by freeze and thawing. GR reaction mixture contained in a final volume of 3 ml, 1,500 μl phosphate buffer, 0.2 M, pH 7.0 containing 2 mM EDTA, 150 μl of 2 mM NADPH in 10 mM Tris-HCl, pH 7.0, 150 μl of 20 mM of GSSG, 1,100 μl of distilled water at 30°C and 100 μl of freshly isolated cellular extracts. The reaction was initiated by the addition of cellular extracts and the decrease in absorbance at 340 nm was followed. GR activity is expressed as the amount of enzyme reducing nanomoles of NADPH per minute per milligram of cellular protein at 30°C ($\epsilon = 6,200 \text{ M}^{-1} \text{ cm}^{-1}$). Proteins were assayed according to the method of Bradford using bovine serum albumin as a reference.

Assay of glutathione peroxidase (GPX) activity

GPX activity was measured according to the method of Yamamoto and Takahashi (1993). Cells (2×10^6) were collected by centrifugation and extracted with 300 μl /dish of RIPA buffer (containing 5 mM EDTA, 0.25% sodium cholate and 0.01% digitonin in PBS.) by freeze and thawing. GPX reaction mixture containing 100 μl of 1 M Tris-HCl buffer, containing 5 mM EDTA (pH 8.0), 20 μl of 0.1 M GSH, 100 μl of 10 U/ml GR, 100 μl of 2 mM NADPH, 580 μl of distilled water and 100 μl of freshly isolated cellular extracts was incubated at 37°C for 2 min. After incubation, the reaction was initiated by the addition of 10 μl of t-BuOH and the decrease in absorbance at 340 nm was followed. GPX activity is expressed as the amount of enzyme reducing nanomoles of GSH per minute per milligram of cellular protein at 37°C.

Assay of glutathione-S-transferase (GST) activity

GST activity was measured according to the method of Habig et al. (1974). Cells (2×10^6) were collected by centrifugation and extracted with 100 μl /dish of 50 mM of potassium phosphate buffer (containing 1 mM EDTA and 0.25 M sucrose adjusted to pH 7.0) by freeze and thawing. GST reaction mixture contained in a final volume of 3 ml, 1,500 μl phosphate buffer, 125 mM, pH 6.5 at 25°C, 150 μl of 1 mM GSH, 300 μl of 1 mM CDNB, 950 μl of distilled water at 25°C and 100 μl of freshly isolated cellular extracts. The reaction was initiated by the addition of cellular extracts and the increase in absorbance at 340 nm was followed. GST activity is expressed as nanomoles of dinitrophenylglutathione formed per minute per milligram of cellular protein ($\epsilon = 9,600 \text{ M}^{-1} \text{ cm}^{-1}$).

Results

Time-dependant effect of ACA on cell viability in EATC

Effect of ACA on cell viability was examined in EATC by trypan blue method. As shown in Fig. 1, ACA decreased cell viability time-dependently.

Effect of ACA on intracellular GSH

Intracellular GSH level, which is often related to cell death, was assayed by HPLC. As shown in Fig. 2, intracellular GSH level decreased by 50% at 15 min after ACA treatment.

Effects of GEE and NAC on cell viability in ACA-treated EATC

To confirm the role of decrease in GSH content in ACA-induced cell death, we used GEE and NAC to elevate the intracellular GSH level simultaneously with the addition of ACA. When cells were incubated with 1 mM GEE or 3 mM NAC, ACA-induced decrease of cell viability was suppressed markedly (Fig. 3). Incubation with GEE alone or NAC alone had no significant action on cell viability compared with control cells (data not shown).

To examine whether GEE can protect the cell against ACA-induced cell death even after ACA exposure, 1 mM GEE was added to the cells at the appropriate time (from 0 to 60 min) after the start of exposure to 40 μM ACA. As shown in Fig. 4, addition of GEE at 0 to 10 min after the start of ACA exposure prevented the decrease in the cell viability.

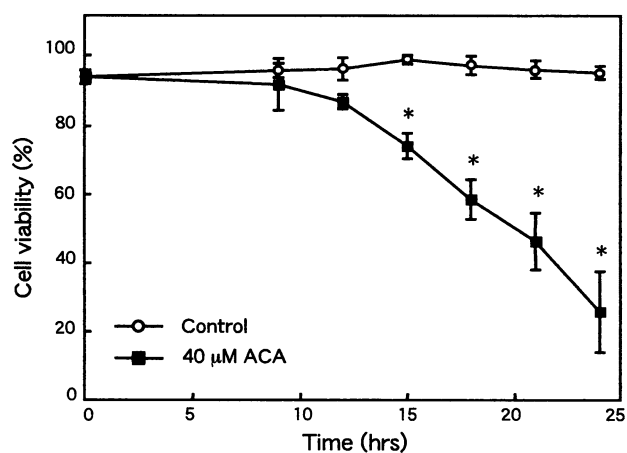


Fig. 1 Time-dependent effect of ACA on cell viability in EATC. Cells were incubated with or without 40 μM ACA for 9, 12, 15, 18, 21 and 24 h. After incubation, cell viability was assayed by trypan blue exclusion. Results are means $\pm$ SD from three experiments. * $P < 0.01$ compared to control cells

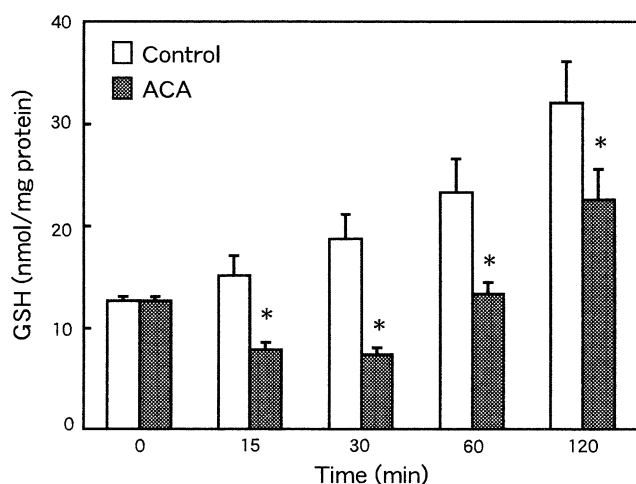


Fig. 2 Time-dependent effect of ACA on cellular GSH. Cells were incubated with or without 40 μ M ACA for 0–120 min. After incubation, cells were collected by centrifuge and lysed by freeze and thawing. The cell lysates were centrifuged at 14,000 $\times$ g for 30 min and the supernatants were used for GSH analysis. Cellular GSH was analyzed by HPLC. Results are means $\pm$ SD from three experiments. * $P < 0.01$ compared to control cells at same time

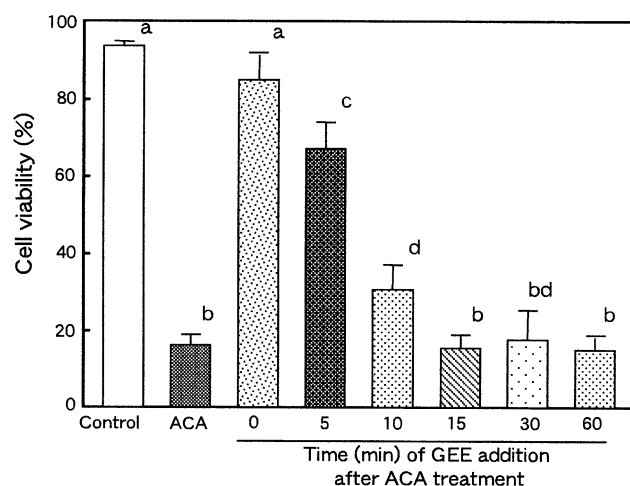


Fig. 4 Time-dependent effect of GEE on cell viability in ACA-treated EATC. Cells were incubated with or without 40 μ M ACA for 24 h and 1 mM GEE was added 0, 5, 10, 30 and 60 min after the ACA treatment. After incubation, cell viability was assayed by trypan blue exclusion. Results are means $\pm$ SD from three experiments. Data not sharing common alphabet are significantly different ($P < 0.01$)

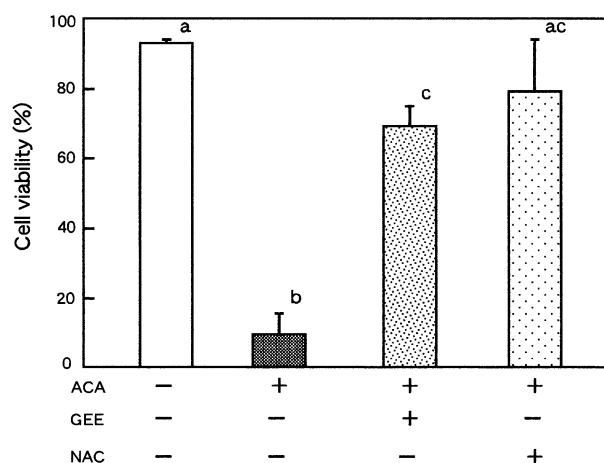


Fig. 3 Effects of GEE and NAC on cell viability in ACA-treated EATC. Cells were incubated with DMSO (0 μ M ACA) or 40 μ M ACA or 40 μ M ACA plus 1 mM GEE or 40 μ M ACA plus 3 mM NAC for 24 h. After incubation, cell viability was assayed by trypan blue exclusion. Results are means $\pm$ SD from three experiments. Data not sharing common alphabet are significantly different ($P < 0.01$)

However, addition of GEE at 15 min after the ACA treatment could not prevent the decrease in cell viability any longer.

Effects of ACA on GSH-related enzyme activities and intracellular gamma-EC

Glutathione reductase is a flavoprotein catalyzing the NADPH-dependant reduction of oxidized glutathione

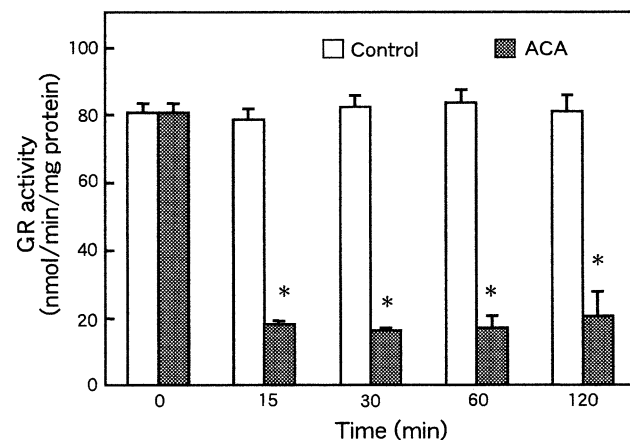


Fig. 5 Time-dependent effect of ACA on GR activity. Cells were incubated with or without 40 μ M ACA for 0–120 min. After incubation, cells were collected by centrifuge and lysed by freeze and thawing. The cell lysates were centrifuged at 30,000 $\times$ g for 15 min and the supernatants were used for GR activity analysis. GR activity was expressed as the amount of enzyme reducing 1 nmol of NADPH per minute per milligram of cellular protein at 30°C. Results are means $\pm$ SD from three experiments. * $P < 0.01$ compared to control cells at same time

(GSSG) to reduced glutathione (GSH). To examine the effect of ACA on the GR activity, cells were exposed to 40 μ M ACA for 0–120 min. As shown in Fig. 5, the cellular GR activity was inhibited by 15 min after ACA exposure by more than 50%. The enzyme inhibition was maintained until 120 min after ACA exposure. To rule out the possibility that ACA directly inhibits the activity of GR, we measured GR activity in vitro in the presence of ACA at various concentrations up to 40 μ M. The enzyme

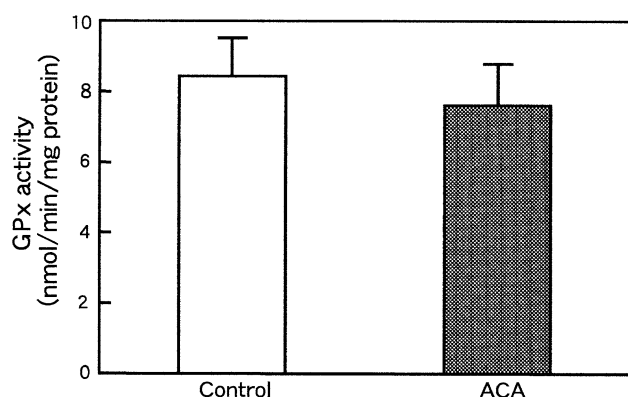


Fig. 6 Effect of ACA on GPX activity. Cells were incubated with or without 40 μ M ACA for 30 min. After incubation, cells were collected by centrifuge and lysed by freeze and thawing. The cell lysates were centrifuged at 14,000 $\times g$ for 30 min and the supernatants were used for GPX activity analysis. GPX activity was expressed as the amount of enzyme oxidizing 1 nmol of GSH per minute per milligram of cellular protein at 37°C. Results are means $\pm$ SD from three experiments

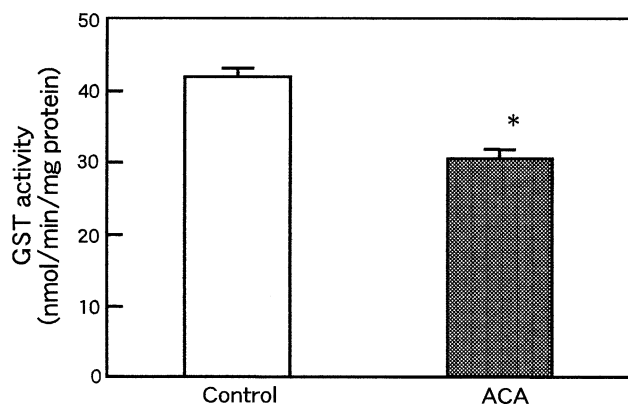


Fig. 7 Effect of ACA on GST activity. Cells were incubated with or without 40 μ M ACA for 30 min. After incubation, cells were collected by centrifuge and lysed by freeze and thawing. The cell lysates were centrifuged at 30,000 $\times g$ for 15 min and the supernatants were used for GST activity analysis. GST activity was expressed as nanomoles of dinitrophenylglutathione formed per minute per milligram of cellular protein at 25°C. Results are means $\pm$ SD from three experiments. * $P < 0.01$ compared to control cells

was not inhibited at all at the concentrations of ACA used (data not shown).

ACA, however, had no significant effect on the activity of Glutathione peroxidase (GPX) (Fig. 6).

Glutathione S-transferase (GST), as detoxification isozymes, protects cells by conjugating GSH to a variety of toxic compounds. In spite of decrease of cellular GSH by ACA-treatment, GST activity was inhibited by ACA exposure (Fig. 7). GSH is synthesized from gamma-glutamyl cysteine (gamma-EC) and glycine by glutathione synthetase (GS). As shown in Fig. 8, in control cells, gamma-EC could not be detected. However, when the cells

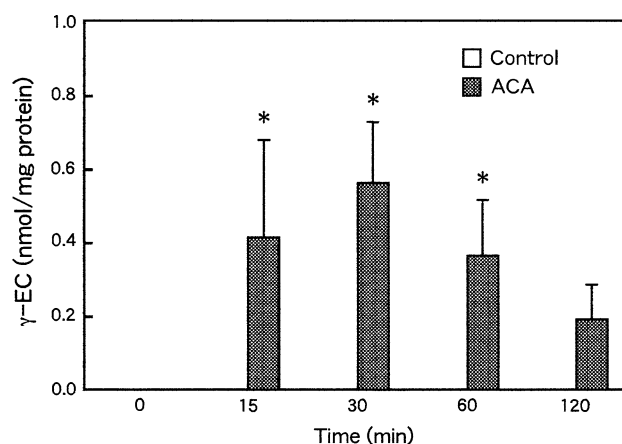


Fig. 8 Time-dependent effect of ACA on cellular gamma-EC. Cells were incubated with or without 40 μ M ACA for 0–120 min. After incubation, cells were collected by centrifuge and lysed by freeze and thawing. The cell lysates were centrifuged at 14,000 $\times g$ for 30 min and the supernatants were used for gamma-EC analysis. Cellular gamma-EC was analyzed by HPLC. Results are means $\pm$ SD from three experiments. * $P < 0.01$ compared to control cells at same time

were incubated with ACA for 0–120 min, intracellular gamma-EC was detected and increased until 30 min after ACA exposure. GSH synthesis seems to be inhibited by ACA exposure.

Effect of ACA on intracellular GSH, GSSG and total GSH

As shown in Fig. 9, ACA decreased not only GSH but also GSSG. The decrease in intracellular GSH and GSSG with ACA was abolished by the addition of GEE or NAC.

Discussion

We showed in a previous study that ACA induced tumor cell death. This cell death was furthermore shown to be apoptotic in nature, as ACA caused modulation of polyamine metabolism and caspase-3-like protease activation (Moffatt et al. 2000), events shown to be characteristics of apoptosis. The cell death was reversed by the addition of NAC or GEE, a thiol supplier. Furthermore, we showed that ACA caused an accumulation of tumor cells in the G1 phase of the cell cycle, which was accompanied by a decrease in phosphorylated retinoblastoma protein (Rb), an increase in Rb and an induced nuclear localization of p27^{kip1}, in its growth inhibitory effect in Ehrlich ascites tumor cells (Unahara et al. 2007). These results showed that ACA caused not only apoptosis but also the suppression of the cell proliferation. On the other hand, we studied the effect of ACA on normal cells using isolated primary

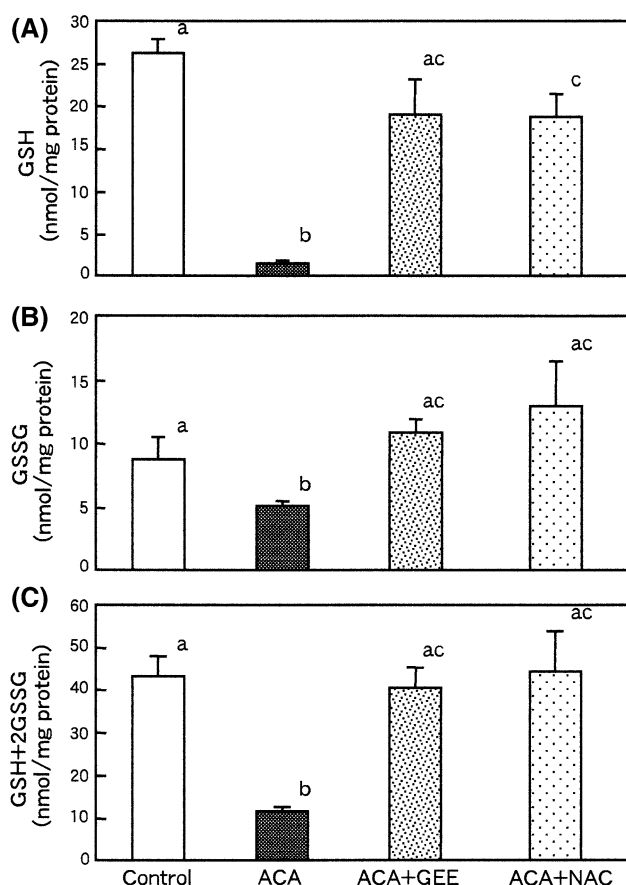


Fig. 9 Effects of NAC and ACA on intracellular GSH (a), GSSG (b) and total GSH (c). Cells were incubated with DMSO (control) or 40 μ M ACA or 40 μ M ACA plus 1 mM GEE or 40 μ M ACA plus 3 mM NAC for 30 min. After incubation, cells were collected by centrifuge and lysed by freeze and thawing. The cell lysates were centrifuged at $14,000 \times g$ for 30 min and the supernatants were used for GSH analysis. GSH was analyzed by HPLC. GSSG was reduced and changed for GSH by TCEP and calculated from total GSH. GSSG was deduced as: $2[\text{GSSG}] = [\text{total GSH}] - [\text{GSH}]$. Results are means $\pm$ SD from three experiments. Data not sharing common alphabet are significantly different ($P < 0.01$)

rat hepatocytes and found that treatment with increasing concentrations of ACA under similar concentration as the tumor cells did not result in any significant changes in cell growth (Moffatt et al. 2000).

The addition of NAC or GEE, a thiol supplier, reversed the ACA-induced cell death. The redox state of the cell has been shown to regulate its growth behavior and several studies have also demonstrated that the onset of apoptosis is associated with a fall of intracellular GSH in different cellular systems (Kannan and Jain 2000). In a previous study, we also showed that ACA was tightly coupled with a number of downstream events in cell cycle G1 arrest, including decrease in the phosphorylation of retinoblastoma protein and p27^{kip1}, an inhibitor of cyclin-dependent kinase, and increases in nuclear localization of p27^{kip1}. NAC and GEE reversed these changes induced by ACA

(Unahara et al. 2007). Our results therefore suggest that in EATC, induction of apoptosis and the suppression of cell proliferation elicited by ACA are dependent on the cellular thiol status. The significant finding in this study is that ACA caused a rapid and significant decrease in intracellular GSH and GSSG after ACA exposure. We demonstrated that ACA treatment depleted GSH content in EATC in less than 10 min of incubation. Debiton et al. (2003) demonstrated that treatment of sanguinarine, the quaternary benzophenan-thridine alkaloid, depleted GSH content in L-929 murine cells (treated with 5 μ M sanguinarine) in less than 10 min of incubation. Ulrichova et al. (2001) reported that the treatment with high concentrations of this drug (25–100 μ M) caused GSH depletion in cultured hepatocytes and the depletion appeared to be correlated with an increased membrane permeability (identified by lactate dehydrogenase enzyme leakage). However, no modification in membrane permeability was detected at low concentrations (2.5, 5 μ M) of sanguinarine. In our experiments, we also measured the leakage of lactate dehydrogenase into the medium from the tumor cells and found that the depletion of intracellular GSH in 40 μ M ACA treated-cells could not be correlated with increased membrane permeability. Therefore, in this study we examined the effect of ACA on GSH-related enzyme activities in EATC. The cellular GR activity was inhibited at a very early stage after ACA exposure and the enzyme inhibition was maintained until 120 min. We also found that gamma-EC, a substrate of GSH synthetase, increased until 30 min after ACA exposure, suggesting that GSH synthetase is inhibited by ACA exposure. The presence of GSH may explain the undetectable levels of gamma-EC in the control cultures as gamma-EC might have been used to produce GSH (Fig. 2). On the other hand as ACA depletes GSH (or inhibit GSH production) there is a detectable level of unused gamma-EC. Therefore GSH synthetase seems to be inhibited by ACA exposure and GSH synthesis is affected by modulations of gamma-EC levels. To know whether ACA has direct effect on GR we measured its activity in the presence of ACA at various concentrations up to 40 μ M in vitro. ACA had no direct effect on the enzyme (data not shown). To date, the detailed mechanism of the inhibition of GR and/or GSH synthetase activities by ACA exposure remains to be investigated. Recent evidence has demonstrated that GSH levels are elevated in various human cancer tissues as compared with normal tissues in the same region (Schneildorfer et al. 2000). Similar evidence has shown that elevated GSH levels in tumor tissues are associated with resistance to chemotherapy. ACA has a chemopreventive effect on chemically induced tumor formation in mouse and rats. The present results showed that ACA caused the decrease in the intracellular GSH levels, suggesting that

ACA-induced reduction of cellular GSH levels can lead to growth arrest of cancer cells and enhancement of the efficacy of other anticancer drugs.

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