Amino Acids (2007) 33: 469–476
DOI 10.1007/s00726-006-0437-4
Printed in The Netherlands

Amino Acids

Cellular thiol status-dependent inhibition of tumor cell growth via modulation of $p27^{kip1}$ translocation and retinoblastoma protein phosphorylation by 1′-acetoxychavicol acetate

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Received February 2, 2006
Accepted September 1, 2006
Published online October 10, 2006;

Summary. 1′-Acetoxychavicol acetate (ACA) has been shown to inhibit tumor cell growth, but there is limited information on its effects on cell signaling and the cell cycle control pathway. In this study, we sought to determine how ACA alters cell cycle and its related control factors in its growth inhibitory effect in Ehrlich ascites tumor cells (EATC). ACA caused an accumulation of cells in the G1 phase and an inhibition of DNA synthesis, which were reversed by supplementation with N-acetylcysteine (NAC) or glutathione ethyl ester (GEE). Furthermore, ACA decreased hyperphosphorylated Rb levels and increased hypophosphorylated Rb levels. NAC and GEE also abolished the decease in Rb phosphorylation by ACA. As Rb phosphorylation is regulated by G1 cyclin dependent kinase and CDK inhibitor $p27^{kip1}$, which is an important regulator of the mammalian cell cycle, we estimated the amount of $p27^{kip1}$ levels by western blotting. Treatment with ACA had virtually no effect on the amount of $p27^{kip1}$ levels, but caused a decrease in phosphorylated $p27^{kip1}$ and an increase in unphosphorylated $p27^{kip1}$ as well as an increase in the nuclear localization of $p27^{kip1}$. These events were abolished in the presence of NAC or GEE. These results suggest that in EATC, cell growth inhibition elicited by ACA involves decreases in Rb and $p27^{kip1}$ phosphorylation and an increase in nuclear localization of $p27^{kip1}$, and these events are dependent on the cellular thiol status.

Keywords: ACA – Tumor cell growth – Rb – $p27^{kip1}$ – Glutathione – Cell cycle

Abbreviations: pRb, hyperphosphorylated Retinoblastoma gene product; Rb, hypophosphorylated Rb; $p27^{kip1}$, CDK inhibitor $p27^{kip1}$; EATC, Ehrlich ascites tumor cells; NAC, N-acetylcysteine; GEE, glutathione ethyl ester

1. Introduction

1′-Acetoxychavicol acetate (ACA), a compound naturally obtained from rhizomes and seeds of South East Asia plants, exhibits chemopreventive effects on chemically induced mouse skin (Murakami et al., 1996) and rat oral (Ohnishi et al., 1996), colon (Tanaka et al., 1997a, b), esophageal (Kawabata et al., 2000) and pancreatic (Miyauchi et al., 2000) tumor formation. The compound prevented glutathione *S*-transferase-positive focal lesions formation elicited by choline-deficient L-amino acid deficient diet (Kobayashi et al., 1998), and caused a reduction in TPA-induced reactive oxygen species in mouse skin (Nakamura et al., 1998). In lipopolysaccharide and interferon-γ stimulated macrophages, ACA markedly suppressed excessive induction of inducible NO synthase through its inhibitory effects on transcription factors such as NF-κB and AP-1 (Ohata et al., 1998). ACA was able to induce apoptosis in Ehrlich ascites tumor cells in vitro through modulation of polyamine metabolism, caspase-3 activation (Moffatt et al., 2000), and protein tyrosine phosphorylation and reduction of cellular sulfhydryl groups (Moffatt et al., 2002). These findings fairly represent mechanisms by which ACA exerts its chemopreventive role.

The retinoblastoma (Rb) gene product is well known as a tumor suppressor and is either absent or mutated in many human tumors. This product is a phosphoprotein (110–116 kDa) that is expressed in most normal cells of vertebrates (Riley et al., 1994), and acts as a tumor suppressor by providing a cell cycle checkpoint between the G1 and S phases (Kawada et al., 1999; Sambucetti et al., 1999), and undergoes differential phosphorylation during

the cell cycle. The active hypophosphorylated form of Rb is primarily associated with resting or fully differentiated cells and becomes increasingly phosphorylated throughout the cell cycle until late mitosis, when substantial dephosphorylation occurs. The hypophosphorylated Rb interacts with a number of cellular proteins including the E2F transcription factor, several cyclins, c-myc and p46. The activity of Rb is negatively regulated by cyclin-dependent kinases (Cdks), which phosphorylate Rb in late G1. Thus the hyperphosphorylated form is primarily found in proliferating cells.

The cyclin-dependent kinase (Cdk) inhibitor $p27^{kip1}$ is an important regulator of the mammalian cell cycle (Hengst and Reed, 1998; Sherr and Roberts, 1995, 1999). $p27^{kip1}$ negatively regulates G1 progression by binding to cyclin-Cdk2 complexes and preventing their activity. Accordingly, in tumors with abundant $p27^{kip1}$ expression, the protein is often mislocalized to the cytoplasm (Baldassarre et al., 1999; Ciaparrone et al., 1998; Singh et al., 1998). Since the growth-restraining activity of $p27^{kip1}$ depends on its nuclear localization, the aberrant compartmentalization of $p27^{kip1}$ probably impairs its function (Baldassarre et al., 1999; Jiang et al., 2000; Orend et al., 1998; Soucek et al., 1998; Yaroslavskiy et al., 1999).

Mechanisms of the modulation of those events that regulate the cell cycle by ACA have not been fully elucidated. In this study, we sought to determine the involvement of Rb and $p27^{kip1}$ phosphorylation and nuclear localization of $p27^{kip1}$ in the growth inhibitory effect of ACA under the hypothesis that ACA could be influencing these important cell cycle factors. Also, we sought to find how cellular thiol status modulates these effects since a previous finding in this laboratory showed that ACA decreases intracellular glutathione levels leading to the regulation of tumor cell growth. Elucidation of the cellular thiol-dependent mechanisms evoked by ACA could provide a useful insight into additional mechanisms governing cell cycle progression during growth arrest by ACA.

2. Materials and methods

2.1 Materials

ACA was isolated from *Languas galanga* as previously reported (Kondo et al., 1993). Glutathione ethyl ester (GEE), was obtained from Sigma (St. Louis, MO, USA). N-acetylcysteine (NAC) was purchased from Wako (Osaka, Japan). Fetal calf serum (FCS) was purchased from JRH Bioscience (Lenexa, Australia). Anti-human Retinoblastoma protein (Rb) antibody was purchased from BD Biosciences. Anti-mouse p27 (M-197) antibody was purchased from Santa Cruz Biotechnology. Biotinylated goat anti-mouse IgG, anti-rabbit IgG and horse radish peroxidase-coupled streptavidin were obtained from DAKO (Kyoto, Japan). Other chemicals used in this study were special grade commercial products.

2.2 Cell culture

Ehrlich ascites tumor cells (EATC) were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C for 3–4 days in Eagle's minimum essential medium containing 10% FCS (JRH Bioscience), washed and cultured again at 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 <0.25%). In all experiments control cultures were made up of medium, DMSO and the cells only.

2.3 Assay of cell viability

Cell viability was determined by Trypan blue exclusion assay. Briefly, cells (1×10^6 cells/ml) were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C in Eagle's minimum essential medium containing 10% FCS for 24 h with or without ACA. NAC (Wako) or GEE (Wako) was added with ACA simultaneously. 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.

2.4 Measurement of cell cycle changes

ACA-induced cell cycle changes were analyzed by Laser Scanning Cytometer using propidium iodide (PI) staining. Briefly, after designated treatments, cells were washed, resuspended in phosphate buffered saline (PBS), and then fixed with ice-cold 70% ethanol at 4 °C for 30 min. The fixed cells were incubated with freshly prepared PI staining buffer (0.1% Triton X-100 in PBS, 50 μg/ml PI, 150 μg/ml RNase) for 1 h at 37 °C.

2.5 Assay of DNA synthesis

Cells were labelled with ^{3}H-thymidine from 23 to 24 h after ACA addition. The labelled cells were washed with PBS and radioactivity of acid-insoluble fraction was measured as the amount of DNA synthesis (Omura et al., 1998).

2.6 Preparation of protein for western blotting analysis of Rb and $p27^{kip1}$

ACA-treated cells were washed twice in PBS and resuspended in 100 μl of lysis buffer (150 mM NaCl, 50 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium deoxycholate, 1% Nonidet P-40, 10 μg/ml pepstatin, 1 mM sodium vanadate, 50 μg/ml leupeptin, 20 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonate, pH 7.5) for 20 min on ice followed by freeze-thawing three times. Cell lysates were centrifuged at 17,500 × *g* for 2 min at 4 °C and supernatant was collected for western blotting. Protein concentrations were determined by the Bradford method (Bradford, 1976).

2.7 Cytosolic and nuclear extracts for western blotting analysis of $p27^{kip1}$

Nuclear extracts were prepared by the method of Staal et al. (1990) with the following modifications. Cells were washed in 8 ml of PBS and centrifuged at 250 × *g* for 5 min at 4 °C. The pellet was resuspended in 1 ml of PBS, transferred into an eppendorf tube, and centrifuged again at 17,500 × *g* for 2 min at 4 °C. PBS was removed, and the cell pellet was resuspended in 400 μl of buffer A (10 mM HEPES, pH 7.8, 10 mM KCl, 0.1 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin, 20 μg/ml aprotinin, 10 μg/ml pepstatin A, 0.1 mM sodium vanadate, 1 mM NaF) by gentle pipetting. The cells were allowed to swell on ice for 15 min, after which 25 μl of 10% Nonidet P-40 was added, and the tube was vortexed vigorously for 10 s. The homogenate was centrifuged at 17,500 × *g* for 5 min at 4 °C and supernatant was collected

as cytosolic extract. The nuclear pellet was resuspended in 60 μl of buffer C (20 mM HEPES, pH 7.8, 0.42 M NaCl, 5 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10% (v/v) glycerol), and the tube was rocked gently for 30 min at 4 °C on a shaking platform. The nuclear extract was centrifuged at 17,500 × *g* for 5 min at 4 °C and supernatant was collected as nuclear extract. Protein concentrations were determined by the Bradford method (Bradford, 1976).

2.8 Western blotting analysis of Rb and p27^{kip1}

Protein (30 μg) from cell lysate for Rb assay or protein (40 μg) from cell lysate, cytosolic extract and nuclear extract for p27^{kip1} assay were loaded onto each lane of a 7.5% (Rb) or 15% (p27^{kip1}) sodium dodecyl sulfate (SDS)-polyacrylamide gel, respectively, and the separated proteins were blotted to 0.45 μm PVDF membrane (Amersham Pharmacia Biotech). After an overnight blocking with 5% non-fat milk, 0.1% Tween-20, in PBS for Rb assay or blocking solution, 3% bovine serum albumin (BSA), 1.5% normal horse serum, in PBS for p27^{kip1} assay, each membrane was stained with anti-Retinoblastoma protein (Rb) antibody (BD Biosciences) or anti-mouse p27 (M-197) antibody (Santa Cruz) for 1 h at room temperature. After washing, the membrane was re-incubated with 1:1500 diluted biotinylated mouse IgG (DAKO) for Rb assay or biotinylated rabbit IgG (DAKO) for p27^{kip1} assay and for 1 h at room temperature. The membrane were washed several times, and then incubated with 1:400 diluted horse radish peroxidase-coupled streptavidin for 1 h at room temperature. After several washing steps the color reaction was developed with 3-amino-9-ethylcalbazole (Arimura et al., 2003; Kennedy et al., 2001; Piwocka et al., 2001; Tanaka et al., 1998). Efficiency of transfer and equal loading of proteins were confirmed by staining membranes with Coomassie blue.

2.9 Statistical analysis

Data are represented as mean ± S.D. (standard deviation from the mean) and statistical evaluations were made using analysis of variance with Fisher's post hoc comparison test. $p < 0.05$ was used to indicate a statistically significant difference.

Table 1. Effect of NAC and GEE on cell viability and DNA synthesis in ACA-treated EATC

	Cell viability %	DNA synthesis dpm/ng DNA (% of control)
Control	92.9 ± 0.7^{a}	26.0 ± 1.1 (100.0 ± 4.1)^{a}
ACA (40 μM)	9.3 ± 5.8^{b}	5.2 ± 1.8 (19.9 ± 6.7)^{c}
ACA (40 μM) + NAC (3 mM)	79.1 ± 12.8^{c}	22.9 ± 2.1 (88.1 ± 8.2)^{b}
ACA (40 μM) + GEE (1 mM)	69.1 ± 5.1^{ac}	26.2 ± 0.5 (100.5 ± 1.9)^{a}

Cells were incubated for 24 h with or without ACA. NAC or GEE was added with ACA simultaneously. After incubation, cell viability was assayed by trypan blue exclusion. DNA synthesis was analysed by incorporation of ^{3}H-thymidine into acid-insoluble fraction. Cells were labeled with ^{3}H-thymidine from 23 to 24 h after ACA addition. Results are means ± S.D. from three experiments. Data not sharing common alphabets are significantly different ($p < 0.05$) using Fisher's test

Table 2. Effect of ACA on cell cycle

	Control	ACA	ACA + NAC	ACA + GEE
Sub-G1 (%)	0.0	11.1	4.6	3.3
G1 (%)	32.7	53.6	21.1	20.1
S (%)	19.1	12.7	24.7	23.8
G2/M (%)	48.2	22.6	49.6	52.8

Cells were treated with 40 μM ACA and cultured for 24 h, harvested, washed, resuspended in PBS, and then fixed with ice-cold 70% ethanol at 4 °C for 30 min. The fixed cells were incubated with freshly prepared PI-staining buffer for 1 h at 37 °C, and staining was measured by laser scanning meter. Results are representative of three separate determinations

3. Results

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

Effect of ACA on cell viability was examined in EATC by trypan blue assay. GEE and NAC, which have been shown to increase intracellular GSH levels effectively, were used to enrich GSH in EATC. To examine whether the viability of ACA-treated cells can be suppressed by supplementing with glutathione, GEE or NAC were added to cells with ACA. When cells were incubated with GEE or NAC, ACA-induced decrease of cell viability was suppressed markedly (Table 1). Incubation with GEE alone or NAC alone had no significant action on cell viability compared with control cells.

3.2 ACA and G1 cell cycle arrest

Whether ACA also affects cell cycle arrest was determined by Laser Scanning cytometric analysis after staining the cells with PI. ACA-treated cells exhibited the expected G1 cell cycle arrest, while NAC and GEE reversed this inhibition (Table 2).

3.3 Effect of ACA on DNA synthesis

To confirm the effect of cell cycle G1 arrest induced by ACA, we examined the effect on the amount of DNA synthesis. As shown in Table 1, ACA inhibited the incorporation of ^{3}H-thymidine into acid-insoluble fraction. The inhibition by ACA was recovered by the addition of NAC or GEE.

3.4 Effect of ACA on Rb phosphorylation

The phosphorylation of Rb during G1 is a growth factor-dependent process that is thought to promote restriction point passage (Planas-Silva and Weinberg, 1997). To determine whether ACA impaired Rb phosphorylation, asyn-

chronously growing cells were treated with ACA, and Rb phosphorylation was monitored by western blotting for electrophoretically distinct phosphorylated Rb species (Fig. 1). ACA decreased hyperphosphorylated Rb (pRb) levels and increased hypophosphorylated Rb (Rb) levels at the 2, 4 and 8 h time points in EATC (Fig. 1A, lanes 5, 7 and 9). These changes were also dose-dependent (Fig. 3B). NAC and GEE abolished the effect of Rb phosphorylation decreased by ACA (Fig. 2).

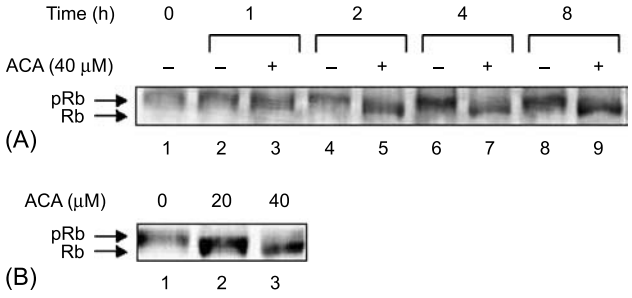

Fig. 1. Effect of ACA on changes in Rb phosphorylation. In time-dependent studies (**A**), cells were cultured in Eagle's minimum essential medium containing 10% FCS with ACA (40 μM) and harvested at indicated times. In dose-dependent studies (**B**), cells were cultured with 20 or 40 μM of ACA and harvested at 8 h. Cell lysates were analyzed by western blotting. *Rb* Hypophosphorylated Rb, *pRb* hyperphosphorylated Rb, are as indicated. Results are representative of three separate determinations

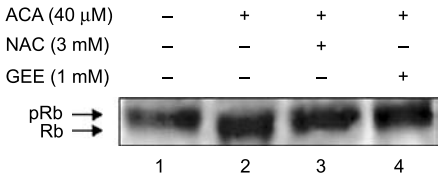

Fig. 2. Effect of NAC and GEE on changes in Rb phosphorylation due to ACA incubation. Cells were cultured in Eagle's minimum essential medium containing 10% FCS with ACA (40 μM), harvested, and lysed at 1 h. Cell lysates were analyzed by western blotting. *Rb* Hypophosphorylated Rb, *pRb* hyperphosphorylated Rb, are as indicated. Results are representative of three separate determinations

3.5 Effect of ACA on p27^{kip1}

Rb phosphorylation is regulated by G1 cyclin dependent kinases (CDKs) and their inhibitors. CDK inhibitor p27^{kip1} is an important regulator of the mammalian cell cycle (Hengst and Reed, 1998; Sherr and Roberts, 1995, 1999). To determine whether ACA affected p27^{kip1} levels, we estimated the amount of p27^{kip1} levels by western blotting. Treatment with ACA had virtually no effect on the amount of p27^{kip1} levels, but caused a decrease in phosphorylated pp27^{kip1} and an increase in unphosphorylated p27^{kip1} (Ishida et al., 2000) (Fig. 3, lanes 3, 5, 7 and 9).

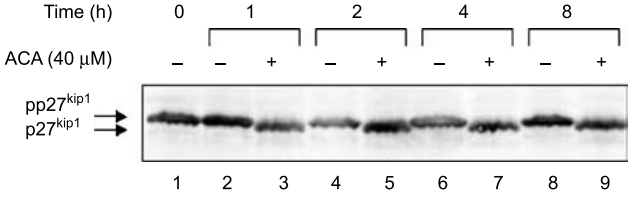

Fig. 3. Time course effect of ACA on p27^{kip1} phosphorylation. Cells were cultured in Eagle's minimum essential medium containing 10% FCS with ACA (40 μM), harvested, and lysed at indicated times. Cell lysates were analyzed by western blotting. Unphosphorylated p27^{kip1} and phosphorylated pp27^{kip1} are as indicated. Results are representative of three separate determinations

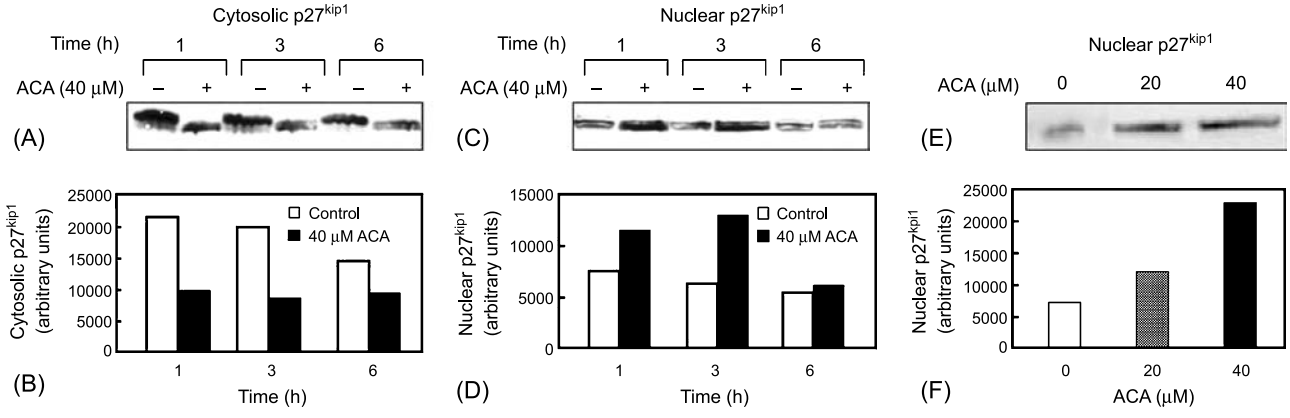

Fig. 4. Effect of ACA on localization of p27^{kip1}. In time-dependent studies (**A**–**D**), cells were cultured in Eagle's minimum essential medium containing 10% FCS with ACA (40 μM) and harvested at indicated times. In dose-dependent studies (**E**, **F**), cells were cultured with 20 or 40 μM of ACA and harvested at 1 h. Cytosolic Extract and Nuclear Extract were made as in Materials and methods. Both extracts were analyzed by western blotting. Cytosolic p27^{kip1} (**A**, **B**) and Nuclear p27^{kip1} (**C**–**F**) are as indicated. **A**, **C**, **E** Western blotting. **B**, **D**, **F** Quantification of p27^{kip1} levels by densitometer. Results are representative of three separate determinations

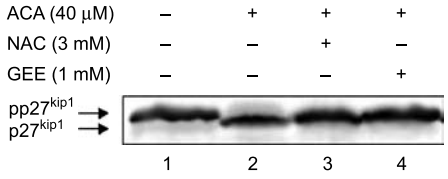

Fig. 5. Effect of NAC and GEE on ACA-induced $p27^{kip1}$. Cells were incubated with DMSO (0 μM ACA) or 40 μM ACA or 40 μM ACA plus 3 mM NAC or 40 μM ACA plus 1 mM GEE, harvested, and lysed at 1 h. Cell lysates were analyzed by western blotting. Unphosphorylated $p27^{kip1}$ and phosphorylated $pp27^{kip1}$ are as indicated. Results are representative of three separate determinations

To exert its inhibitory action, $p27^{kip1}$ needs to be transported into the nucleus (Orend et al., 1998; Reynisdottir and Massague, 1997; Soucek et al., 1998; Tomoda et al., 1999). We thus examined the localization of $p27^{kip1}$ in EATC. In ACA-treated cells, more $p27^{kip1}$ was located in the nucleus than that of control (Fig. 4). Nuclear localization of $p27^{kip1}$ occurred in a dose-dependent manner (Fig. 4). NAC and GEE abolished the decrease in $p27^{kip1}$ phosphorylation by ACA, following the increase in nuclear localization of $p27^{kip1}$ (Figs. 5 and 6).

4. Discussion

The significant finding in this study is that ACA caused an accumulation of tumor cells in the G1 phase of the cell cycle, which was accompanied by a decrease in pRb, an increase in Rb and an induced nuclear localization of $p27^{kip1}$ in its growth inhibitory effect in Ehrlich ascites tumor cells. These events were all cellular thiol-dependent.

In previous studies, by utilizing the same concentration of test agent and under similar cell culture conditions as in this study, ACA induced tumor cell death ($p<0.05$). 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. Furthermore, adding NAC or GEE, a thiol supplier, reversed the ACA-induced cell death (Table 1).

Many phytochemicals possess the ability to induce cell-cycle G1 arrest (Agarwal et al., 2000; Ahmad et al., 1997; Kennedy et al., 2002; Pan et al., 2002; Shan et al., 1999). Our study demonstrated that ACA also caused growth arrest in tumor cells by eliciting arrest in the G1 phase of the cell cycle (Tables 1 and 2).

In G1-S checkpoint control, Rb plays a critical role where hypophosphorylated Rb prevents cellular proliferation by binding with E2Fs and inhibiting cell cycle progression (Paggi et al., 1996; Wang et al., 1994). Phosphorylation of Rb by CDK/cyclin complex results in the release of active E2F species to stimulate the transcription of genes involved in DNA synthesis and S-phase progression (Hiebert 1993; Qian et al., 1992; Qin et al., 1992). In this study, we observed that ACA decreases hyperphosphorylated Rb (pRb) levels and increases hypophosphorylated Rb (Rb) levels at the 2, 4 and 8 h time points in EATC (Fig. 1A, lanes 5, 7 and 9). These findings imply that cell cycle arrest and growth inhibition in EATC by ACA are associated with decreased Rb phosphorylation.

Kinase activity associated with two G1 cyclins, D and E, is essential for cell cycle S phase progression, predominantly because of the requirement for phosphorylation of Rb and the consequent termination of its inhibition of cell cycle progression (Morgan 1995; Sherr, 1996). $p27^{kip1}$ negatively regulates G1 progression by binding to cyclinE-Cdk2 complexes and preventing their activity. The activity of $p27^{kip1}$ is regulated by its subcellular compartmentalization. One of the key mechanisms involved in

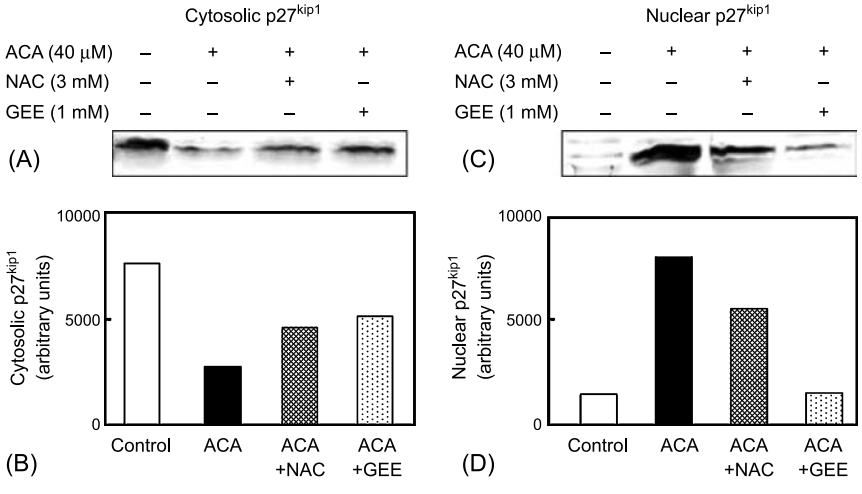

Fig. 6. Effect of NAC and GEE on ACA-induced $p27^{kip1}$ translocation. Cells were incubated with DMSO (0 μM ACA) or 40 μM ACA or 40 μM ACA plus 3 mM NAC or 40 μM ACA plus 1 mM GEE, harvested, and lysed at 1 h. Cytosolic Extract and Nuclear Extract were made as in Materials and methods. Both extracts were analyzed by western blotting. Cytosolic $p27^{kip1}$ (**A**, **B**) and Nuclear $p27^{kip1}$ (**C**, **D**) are as indicated. **A**, **C** Western blotting, **B**, **D** Quantification of $p27^{kip1}$ levels by densitometer. Results are representative of three separate determinations

the regulation of $p27^{kip1}$ abundance is proteolysis by the ubiquitin-proteasome pathway (Pagano et al., 1995). Phosphorylation of $p27^{kip1}$ on threonine 187 (T187) by Cdk2 creates a binding site for a Skp2-containing E3 ubiquitin-protein ligase, SCF (Feldman et al., 1997; Skowyra et al., 1997), and ubiquitylation of $p27^{kip1}$ by SCF results in degradation of $p27^{kip1}$ by the proteasome (Carrano et al., 1999; Sutterluty et al., 1999; Tsvetkov et al., 1999).

To interact with its targets (Cdk2 complexes) and consequently inhibit G1 progression, $p27^{kip1}$ needs to be transported into the nucleus. It is shown that $p27^{kip1}$ is efficiently degraded in the nucleus and phosphorylation of Ser10 is necessary for the nuclear to cytoplasmic re-distribution of a fraction of $p27^{kip1}$ in response to mitogenic stimulation (Rodier et al., 2001). This cytoplasmic localization may serve to decrease the abundance of $p27^{kip1}$ in the nucleus below a certain threshold required for activation of cyclin-Cdk2 complexes. Boehm et al. (2002) have shown that hKIS is a nuclear protein that binds the C-terminal domain of $p27^{kip1}$ and phosphorylates it on S10 in vitro and in vivo, promoting its nuclear export to the cytoplasm. Furthermore, recent studies have suggested that Akt-induced T157 phosphorylation causes retention of $p27^{kip1}$ in the cytoplasm, precluding $p27^{kip1}$-induced G1 arrest (Viglietto et al., 2002). Immunoblot analysis with anti-$p27^{kip1}$ in EATC revealed that these antibodies recognized two types of bands, suggesting that the lower mobility band might correspond to phosphorylated $p27^{kip1}$ (Fig. 3, lanes 1, 2, 4, 6 and 8; lanes 3, 5, 7 and 9; the two types of bands may be a result of a difference in composition of the acrylamide gel). In ACA-treated cells, more $p27^{kip1}$ was located in the nucleus than that of control (Fig. 4). These data suggest that ACA exerts its growth-inhibitory effects through prevention of phosphorylation and nuclear export of $p27^{kip1}$ to the cytoplasm.

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). Inhibition of cell cycle progression by GSH depletion in cancer cells has also been reported (Liu et al., 2000; Vahrmeijer et al., 1999), and GSH has been regarded as an important cellular thiol and the major determinant of the intracellular redox potential (Kennedy et al., 1999; Hall, 1999; Liu et al., 2000). We had previously shown that a decrease in GSH and protein-SH was elicited by ACA and this was tightly coupled with a number of downstream events in apoptosis (Moffatt et al., 2002). In this study, ACA was tightly coupled with a number of downstream events in cell cycle G1 arrest, including decreases in Rb and $p27^{kip1}$ phosphorylation and increases in nuclear localization of $p27^{kip1}$. NAC and GEE reversed these changes induced by ACA (Figs. 2, 5 and 6). NAC has been shown to act as an antioxidant and also as a sulfhydryl group supplier. Anderson and Meister (1989) showed that GEE enters cells where it is converted to free glutathione, thus raising intracellular GSH levels. Our results therefore suggest that in EATC, cell growth inhibition elicited by ACA involves decreases in Rb and $p27^{kip1}$ phosphorylation and increases in nuclear localization of $p27^{kip1}$, and these events are dependent on the cellular thiol status. Recent evidence has demonstrated that GSH levels are elevated in various human cancer tissues as compared with normal tissues in the same region (Schnelldorfer et al., 2000). Similar evidence has shown that elevated GSH levels in tumor tissue are associated with resistance to chemotherapy. This cellular thiol-dependent mechanism evoked by ACA may provide a useful insight into additional mechanisms governing cell cycle progression during growth arrest by ACA.

Acknowledgement

This study was conducted with funds from Japan Society for the Promotion of Science and is gratefully acknowledged.

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