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Aristolochic acid-induced cell cycle G1 arrest in human urothelium SV-HUC-1 cells

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Abstract

Aristolochic acid (AA) has been implicated in urothelial carcinoma in humans. To evaluate the impact of AA on the human urinary tract epithelium cells, a study of SV-HUC-1 cells cultured with mixture of AA (AAM; 41% AA I, 56% AA II) was conducted. Cell viability was assayed in cultures exposed to 0.0125–0.2 mM AAM for 1, 3, and 5 days, a concentration-dependent inhibition on the growth of SV-HUC-1 cells was demonstrated. Cell cycle distribution determined by flow cytometry revealed an accumulation of cells in the G0/G1 phase (from 37.6% to 49.2%). Regarding the cell cycle control proteins, the levels of p53, p21 and p27 increased in a concentration-dependent manner. Immunoprecipitation demonstrated a decrease in the formation of cyclin E/cdk2 complex, but not cyclin D1/cdk4 complex, which leads to an increase in the free form of cdk2. Additionally, a decrease in the phospho-Rb correlates with an increase in Rb/E2F-1 complex which prevents the release of E2F transcription factor, thus preventing the transcription of the genes required for cell proliferation. Our results provide evidence that AAM induce cell cycle arrest in SV-HUC-1 cells. Whether this cell cycle block is associated with AA-related human urothelial carcinoma requires further study to clarify.
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Keywords: Aristolochic acid; Cell cycle arrest; Urothelium

1. Introduction

Aristolochic acid (AA) extracted from genus *Aristolochia* has been shown to be a genotoxic mutagen (Robisch et al., 1982; Schmeiser et al., 1986) and a potent carcinogen to both rats (Mengs et al., 1982; Schmeiser et al., 1990; Rossiello et al., 1993; Cosyns et al., 1998) and humans (Cosyns et al., 1999; Nortier et al., 2000). Toxicological studies of AA have also given information on its nephrotoxicity (Jackson et al., 1964; Peters and Hedwall, 1963). Clinically, in 1991, AA in slimming capsules was suspected of causing the so-called Chinese herb nephropathy, a progressive renal fibrosis that was diagnosed in a group of young Belgian women who had all followed the same slimming regimen (Vanherweghem et al., 1993; Vanhaelen et al., 1994). Since then, several clinical presentations of AA-related nephropathy have been described from Spain, France, UK, and Taiwan (Pena et al., 1996; Stengel and Jones, 1998; Lord et al., 1999; Yang et al., 2000; Arlt et al., 2002).

Accumulating evidence has demonstrated that the mutagenic and carcinogenic properties of AA are based on the formation of DNA adducts (Arlt et al., 2001). Following reductive metabolic activation, both AAs react with DNA preferentially at the exocyclic amino groups of

Abbreviations: AA, aristolochic acid; AAM, aristolochic acid mixture; cdk, cyclin-dependent kinase; Rb, retinoblastoma; SV-HUC-1, human urinary tract epithelium cell.

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adenine and guanine (Pfau et al., 1990, 1991). The DNA adducts (e.g., 7-(deoxyadenosin-N^6-yl)aristolactam I or II and 7-(deoxyguanosin-N^2-yl)aristolactam I or II) have been detected in the kidney and ureter tissues of patients taking herbs containing AAs, several months or even years after cessation of the herbal consumption. The former was the most predominant DNA adduct in human and rat tissues (Pfau et al., 1990; Schmeiser et al., 1996).

Since Taiwan is a high-incidence area of urinary tract transitional cell carcinoma (Wu et al., 2004), and intake of the AA-containing Chinese herbs is the potential risk for urothelial carcinogenesis. We therefore conducted this study of SV-HUC-1 cells cultured with mixture of AA (AAM; 41% AA I, 56% AA II) to evaluate the impact of AAM on the growth of human urinary tract epithelium cells and the changes in the cell cycle.

2. Materials and methods

2.1. Chemicals

Tris–HCl, EDTA, SDS, phenylmethylsulfonyl fluoride, bovine serum albumin (BSA), leupeptin, Nonidet P-40, deoxycholic acid, sodium orthovanadate aprotinin, RNase A, proteinase K, DNase, polyclonal antibody against α-tubulin and AA mixture (AAM) (41% AA I, 56% AA II) were purchased from Sigma chemical Co. (St. Louis, MO). Protein assay kit was obtained from Bio-Rad Labs. (Hercules, CA). Has's F12 medium (F12-K), fetal bovine serum, penicillin–streptomycin mixed antibiotics, L-Glutamine, Dulbecco's Phosphate Buffer Solution (PBS), sodium pyruvate, and trypsin–EDTA, were purchased from Gibco/BRL (Gaithersburg, MD). Antibodies against cytochrome *c*, and caspase3 were from Santa Cruz (CA, USA), and those against cyclin D1, cyclin E, cdk2, cdk4, p53, p21, p27, Rb and E2F were from Cell Signaling Tech. (MA, USA). The enhanced chemiluminescence (ECL) kit was purchased from Amersham Life Science (Amersham, UK).

2.2. Cell line

The SV-HUC-1 cell line was purchased from the Bioresource Collection and Research Center (BCRC), Taiwan. The cell number was BCRC 60358, originally obtained from the American Type Culture Collection (ATCC) (ATCC number: CRL-9520) and derived from transformed normal human urinary tract epithelial cells in vitro after infection with simian virus 40 (SV40) (Christian et al., 1987). This cell line has been tested in toxicological studies previously (Hirao et al., 1993; Mills et al., 2000; Su et al., 2006). The cells were grown in Has's F12 medium (supplemented with 7% v/v fetal bovine serum, 4 mM L-glutamine, 100 μg ml^{-1} penicillin, and 1 μg ml^{-1} streptomycin).

2.3. Assessment of cell viability

SV-HUC-1 cells (10^5 cell ml^{-1}) were treated with AAM. The culture was exposed to the indicated concentration of AAM (0, 0.025, 0.05, 0.1, 0.2 mM) for 1, 3, and 5 days. Subsequently, the medium was removed and replaced with 3-(4,5-dimethylthiazol-2-xl)-2,5-diphenyltetrazolium bromide [MTT, 0.1 mg/ml] for 4 h. The numbers of viable cells were directly proportional to the production of formazan, which was solubilized in isopropanol and measured spectrophotometrically at 563 nm.

2.4. Flow cytometric analysis

Cells were incubated in fresh medium containing 7% v/v FBS to allow the progression through the cell cycle. At various time periods after relief of the quiescent state, cells were analyzed for cell cycle distribution by flow cytometry. Following various treatments, SV-HUC-1 cells were treated with trypsin and RNase and then stained propidium iodide (PI). Cell cycle distribution is presented as the number of cells versus the amount of DNA as indicated by the intensity of fluorescence, and the extent of apoptosis was determined by counting cells of DNA content below the Sub-G1 peak. The experiment was independently repeated three times and the representative histograms are shown.

2.5. Electrophoresis and immunoblotting

Analysis of cyclin D1, cyclin E, cdk2, cdk4, p53, p21, p27, Rb and E2F were performed with the method of SDS-PAGE and immunoblotting. For the analysis of the expression of proteins, the AAM (0.025–0.1 mM) were added to the culture for 24 h. The medium was removed and rinsed with PBS at room temperature. Then, 0.5 ml of cold RIPA buffer with freshly added leupeptin (10 μg/ml) and sodium orthovanadate (100 mM) was added. Scraping of cells and the transfer of lysate into an eppendorf was performed prior to incubation for 30 min on ice and the addition of 5 μl of 10 mg/ml PMSF stock. Microfuge (10,000*g*) of cell lysate was performed for 10 min at 4 °C. Cell lysate (0.5 μg purified protein) was mixed with an equal volume of electrophoresis sample buffer and then boiled for 10 min, followed by analysis using SDS-PAGE and transfer of protein from the gel to nitrocellulose membranes using immunoblotting apparatus. Thereafter, blocking of non-specific binding is soaking membrane in polyclonal antibody at 1 μg/ml in PBS for 1 h with PBS washing in between. Incubation with Horseradish Peroxidase conjugate anti-mouse or anti-rabbit IgG diluted to 1:2000 in PBS was followed by washing three times for 10 min. Finally, the membrane was incubated in ECL reagents for 1 min and exposed to ECL hyperfilm in a darkroom for 5–10 min.

2.6. Immunoprecipitation

Cell lysates were prepared using lysis buffer containing 50 mM Tris, 5 mM EDTA, 150 mM sodium chloride, 1% Nonidet P-40, 0.5% deoxycholicacid, 1 mM sodium orthovanadate, 81 μg/ml aprotinin, 170 μg/ml leupeptin, and 100 μg/ml phenylsulfonyl fluoride; pH7.5. Five hundred μg of protein from cell lysates was pre-cleared with protein A-Sepharose (Amersham Pharmacia Biotech), followed by immunoprecipitation using monoclonal anti-cdk2, -cdk4, and-E2F (Santa Cruz Biotech) antibodies. Immune complexes were harvested with protein A, and immunoprecipitated proteins were analyzed by SDS-PAGE. Immunodetection was carried out using monoclonal anti-cdk2, -cdk4, -E2F, and -cyclin D1;polyclonal anti-cyclin A, -cyclin E, and -Rb antibodies. Three independent experiments were performed to detect each protein expression in AAM-treated cells in relation to controls.

2.6.1. Statistical analysis

Data have been expressed as mean ± standard deviation (SD). Statistical analysis of cell viability was determined using an unpaired *t*-test. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. AAM inhibited the growth of SV-HUC-1 cell and induced G0/G1 cell cycle arrest

In this study, we demonstrated that AAM induced significant growth inhibition of SV-HUC-1 cells. Cell viability was assayed in cultures exposed to 0.0125–0.2 mM AAM for 1, 3, and 5 days. AAM showed a concentration-dependent inhibition on the growth of SV-HUC-1 cell (Fig. 1). Because AAM inhibited cell growth, we analyzed the effect of this compound on cell cycle distribution by flow

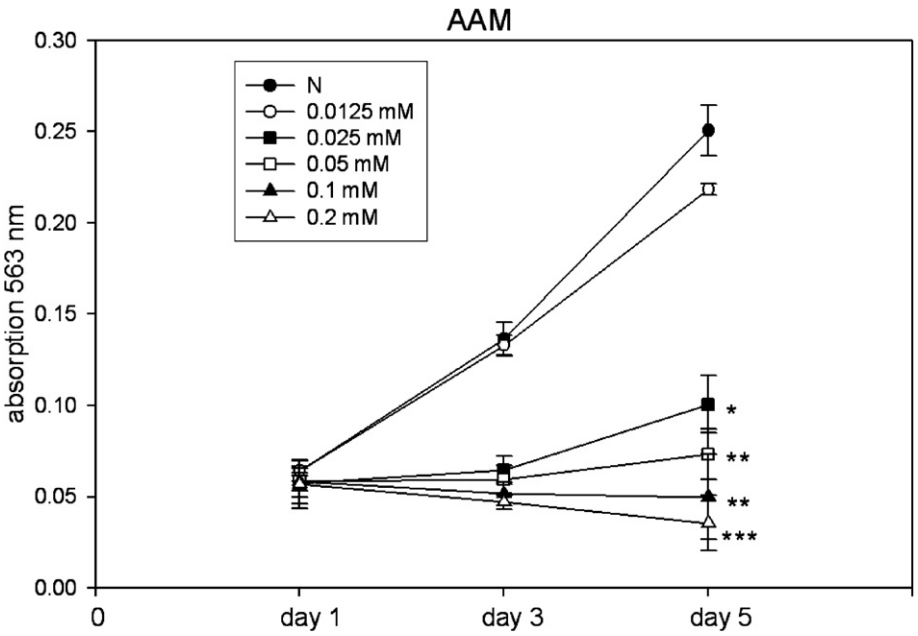

Fig. 1. Dose–response of the viability of SV-HUC-1 cells following treatment with AAM. After overnight attachment of cells, the culture was exposed to indicated concentration of AAM for 1, 3, 5 days. Then the medium was removed and the viability was detected by MTT assay. The viable cell number is directly proportional to the production of formazan. Results are shown as mean ± SD from three independent experiments. * indicates that $p < 0.05$, ** indicates that $p < 0.001$, *** indicates that $p < 0.0001$ compared to control cells.

cytometry. The cells were treated with various concentrations (0, 0.025, 0.05, and 0.1 mM) of AAM for 48 h and collected for analysis of cell cycle distribution. The representative histograms are shown in Fig. 2, AAM caused an apparent accumulation of cells in the G0/G1 phase (from 37.6% to 49.2%).

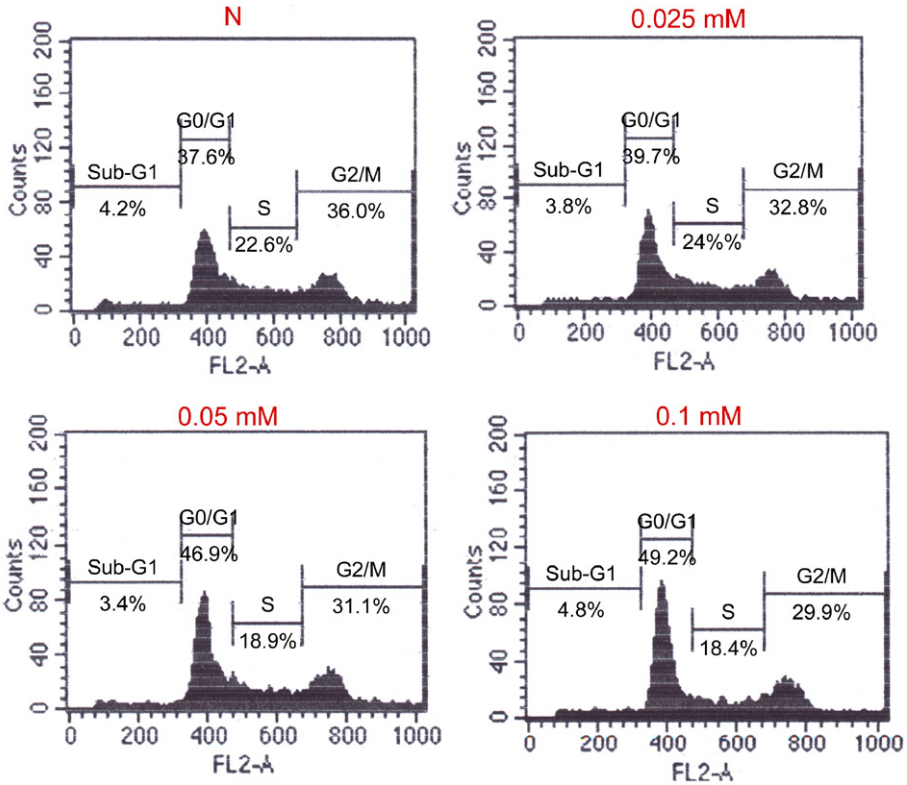

Fig. 2. Effect of AAM-induced cell cycle G0/G1 arrest in SV-HUC-1 cells. Flow cytometric DNA fluorescence profiles of SV-HUC-1 cells. PI-stained DNA histograms of AAM-treated cells are shown. Cells were an untreated control or treated with an indicated concentration of AAM for 48 h. This figure is a representative of three independent experiments with similar results.

3.2. AAM-induced G1 cell cycle regulatory proteins in SV-HUC-1 cells

Different regulators working in multiple pathways tightly regulate cell cycle control, including pRb, cyclin, and cyclin-dependent kinase (cdk) proteins. To investigate the mechanism of AAM-induced G1 checkpoint, SV-HUC-1 cells treated with AAM for 24 h were subjected to immunoblotting analysis. Among several cell cycle control proteins, the levels of p53, p21 and p27 were increased by the treatment of AAM in a concentration dependent manner (Fig. 3). In addition, the cdk inhibitor p27 is also induced by AAM, thereby regulates the G1–S transition. On the contrary, there was no change in the protein expression of cyclin

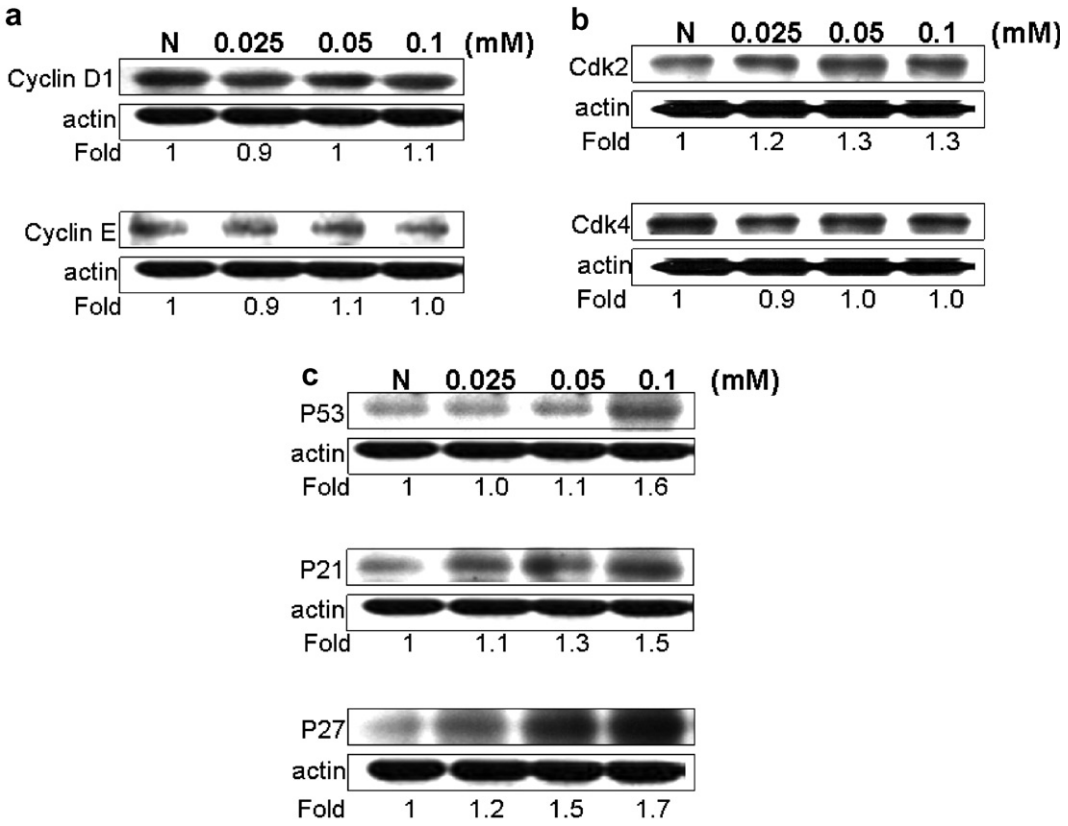

Fig. 3. The levels of protein expression were analyzed after SV-HUC-1 cells treated with AAM by Western blot assay. SV-HUC-1 cells were treated with AAM (24 h) for the indicated concentration and analyzed by immunoblotting with anti-cycin D1, and cycin E antibody (a); anti-cdk2 and cdk4 (b); anti-p53, p21 and p27 (c). Actin was used as the internal control. All these data represented one of three independent experiments.

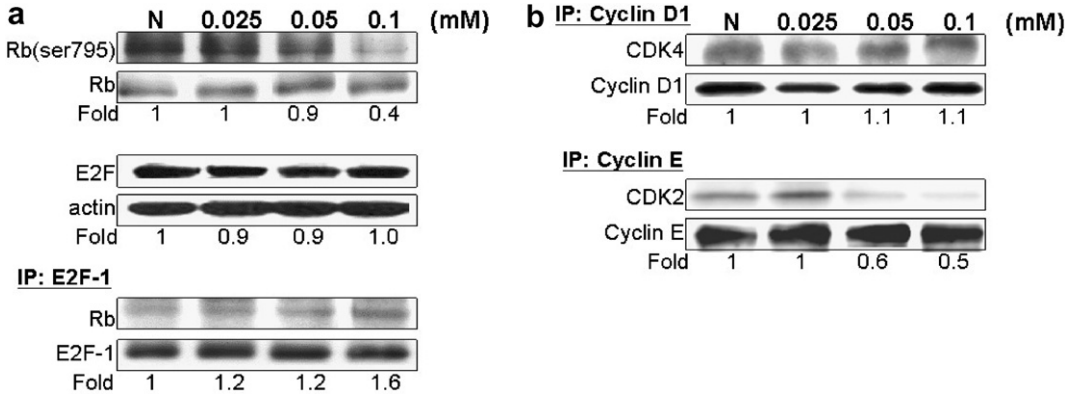

Fig. 4. The effect of AAM on cell cycle regulatory proteins. Following treatment with indicated concentration of AAM, SV-HUC-1 cells were harvested and cell lysates were subjected into western blot analysis using anti-phosph-RB or RB antibody (upper panel); anti-E2F or anti-actin antibody (middle panel). Cell lysates were immunoprecipitated with anti-E2F-1 antibody and performed immunoblotting using anti-Rb and anti-E2F-1 antibodies (lower panel) (a). Cell lysates derived from AA-treated cells were immunoprecipitated with anti-cyclin D1 (upper panel) or anti-cyclin E (lower panel). The immunocomplexes were immunoblotted with anti-CDK4, anti-cyclin D1, anti-CDK2 or anti-cyclin E antibodies (b). All these data represented one of three independent experiments.

D1, cyclin E, and cdk4. However, a significant induction of cdk2 expressions was observed in the treated cells. This phenomenon could have resulted from a reduction in cyclin E/cdk2 complex that leads to an increase in the free form of cdk2. Therefore, we analyzed the level of cyclin E/cdk2 complex by immunoprecipitation. In agreement with the supposition, AAM treatment decreased the formation of cyclin E/cdk2 complex (Fig. 4b) but not cyclin D1/cdk4 complex. Taken together, we suggested that the AAM-induced cell cycle arrest at the G0/G1 phases is associated with the cyclin E/cdk2 proteins.

3.3. AA-induced G1 arrest affects the pRb phosphorylation, E2F-1 protein expression

The phosphorylation of Rb protein mediated by cyclin E/cdk2 is necessary for cells to progress from the G1 to the S phase. To elucidate the role of Rb in the AAM-induced G1 arrest, we assessed the phosphorylated state of Rb and its complex with E2F-1. The results showed that the expression of Rb protein was only slightly increased, although the level of phospho-Rb (Ser795) was decreased by the treatment of 0.1 mM AAM. A decrease in the phospho-Rb is associated with an increase in Rb/E2F-1 complex in the cell cycle. Using immunoprecipitation, we confirmed that the addition of AAM regulated the formation of Rb/E2F-1 complex in SV-HUC-1 cells (Fig. 4a). An increase in the Rb/E2F-1 complex prevents the release of E2F transcription factor and, thus, prevents the transcription of the genes required for cell proliferation. These results indicated that Rb/E2F-1 complex was involved in the AAM-induced G1 arrest.

4. Discussion

AA has been implicated in nephrotoxicity and carcinogenesis in humans. The nephrotoxic effect was shown as early as 1964 (Jackson et al., 1964) and was again noted in 1993 in Belgium because one group of young female patients taking a slimming regimen including Chinese herbs containing AA suffered from rapidly progressing interstitial renal fibrosis (Vanherweghem et al., 1993). In 1994, two of these Belgian AAN patients were reported to have urothelial cancer (Cosyns et al., 1994; Vanherweghem et al., 1995). Thereafter, an increasing number of urothelial carcinomas were reported (Cosyns et al., 1999). In 2000, Nortier et al. reported that the prevalence of urothelial carcinoma among these patients with end stage AAN is rather high, with 46% urothelial carcinoma (18 in 39 patients), 49% urothelial dysplasia (19 in 39 patients) and only 5% (2 in 39 patients) with normal urothelium. The cumulative dose of *Aristolochia fangchi* and the dose of AA were associated with a significantly higher risk of developing urothelial cancer (Nortier et al., 2000). Patients with a mean intake of 200 g of Chinese herbs had a 50% higher risk of developing urothelial cancer.

A hallmark of final differentiation is an irreversible arrest at the G0/G1 phase of the cell cycle. This arrest is associated with a negative control of the cell cycle machinery that inhibits the G1–S transition. A deregulation of the delicate control is a general characteristic of tumor development. Several studies have reported that cell signaling pathways determine the growth as well as the arrest of cells via cell cycle regulation (Agarwal, 2000). Cyclin/cdks complexes control cell cycle progression through their ordered activation and inactivation, and may play an important role in the pathogenesis of various human malignancies (Itoi et al., 2000). For example, cyclin D1 binds to cdk4 and forms a complex that phosphorylates several substrates, including Rb, and thus the transcriptionally activates E2F target genes that trigger cells to progress from G0/G1 to S phase (Dyson, 1998). During the progression of the cell cycle, the cyclin/cdk complexes are inhibited by the binding of CKIs, such as p21, p27, and INK4 families' proteins (Vidal and Koff, 2000). Several studies demonstrated deregulation of cell cycle regulatory proteins is associated with carcinogenesis of various cancers including esophageal squamous cell cancer (Du et al., 2004), gallbladder carcinoma (Itoi et al., 2000), oral tongue squamous cell carcinoma (Wang et al., 2006), breast cancer (Weroha et al., 2006) or Epstein–Barr virus associated gastric carcinoma (Chang et al., 2005), etc. The individual mechanism of cell cycle regulation between different malignancies differs, such as in endometrial clear cell adenocarcinoma and in endometrioid adenocarcinoma (Arai et al., 2006). Additionally, some cell cycle regulators are associated with progression of esophageal squamous cell carcinoma (Chang et al., 2005) or associated with the 10-year survival of patients with ovarian cancer (Terauchi et al., 2005). In contrast, in bladder carcinogenesis, the level of the cell cycle regulators could not predict early recurrence and tumor progression (Ioachim et al., 2004).

The results of present studies, AAM treatment caused a decrease in the levels of cyclin E/cdk2 complex while no changes in the levels of cyclin D1 and cyclin E. Additionally, there was a marked increase of p53, p21, p27 in the SV-HUC-1 cells treated with AAM, indicating that this might be a major molecular mechanism through which an inhibitory effect on the growth of SV-HUC-1 cells.

The most widely studied checkpoint is at G1, although cell cycle arrest can also occur at the S phase and G2/M transition. Following exposure to genotoxic agents, levels of the tumor suppressor protein p53 are frequently elevated, and this upregulates transcription of p21 (el-Deiry et al., 1994), an inhibitor of cyclin E/cdk2 complex, resulting in pRB hypophosphorylation. This favors pRB remaining bound to E2F, which prevents E2F from inducing cells to transition to S phase thus precipitating cycle arrest in the early G1 phase. In addition, the cdk inhibitor p27 plays a critical role in cell cycle regulation by binding and inhibiting (or activating) various cdk/cyclin complexes. The present study showed by flow cytometry a dose-dependent G1 arrest in SV-HUC-1 cells following treatment with AAM.

Consistent with this functional outcome, levels of p53, p21, p27 were increased in cells treated with AAM under various culture conditions. A decrease in the activity of cyclin E/cdk2 complex was induced by the increase of p53, p21 and p27 to repress Rb phosphorylation.

A key regulator of the G1 to S phase transition in the cell cycle is retinoblastoma (Rb), a tumor suppressor protein (Adams, 2001). During the cell cycle progression, Rb is sequentially phosphorylated by different cyclin/CDK complexes (Harbour et al., 1999). Studies have demonstrated that Rb is phosphorylated in a cell cycle-dependent manner, and that progression of cell through G1 into S phase transition requires inactivation of Rb by phosphorylation. The present observation demonstrated AAM down-regulated the phosphorylation of Rb on Ser795, presumably because of a decreased in the cyclin E/cdk2 complex. The level of Rb/E2F-1 complex was subsequently elevated and resulted in cell growth suppression.

Recently, high cumulative incidences of urinary tract transitional cell carcinoma were reported in China and Taiwan (Wu et al., 2004; Li et al., 2005), both reported that intake of the AA-containing Chinese herbs was the potential risk factor for development of urothelial carcinoma. Since the import and the production of AA-containing herbs have been prohibited by Taiwan government since 2003, the incidence of patients with AA-associated nephropathy or urothelial carcinoma might be predicted to be gradually reduced under this policy. Therefore, more basic or clinical studies are needed especially in these years to clarify the AA-related nephropathy and carcinogenesis in Taiwan.

An important limitation to this study regarding the role of p53 and of p53-induced p21 should be addressed. As shown in Fig. 3c, the expression of p53 was not increased if the AAM concentration was less than 0.05 mM. However, p21 could be induced at a lower AAM concentration as compared with the expression of p53. Moreover, the growth inhibitory effects of AAM were detectable at 0.025 mM in parallel with undetectable p53 induction. Therefore, the possibility of a p53-independent effect could not be excluded (Fig. 5). Bartek and Lukas indicated that the TGF-β-activated Smad protein complexes are first thought to mediate potent transcriptional induction of two major cell cycle inhibitors, p15 and p21. Increased levels of these two inhibitors result in a major inhibition of CDK activities associated with the early G1 phase progression, thus locking the cell cycle prior to the G1 restriction point (Bartek and Lukas, 2001). Besides, DNA damage field have recently provided some novel insights into the cell cycle response. DNA damage induced kinase cascades involving ATM/ATR and Chk1–Chk2 target Mdm2 or p53 to induce p21 protein expression, and delay the G1/S phase (Moustakas et al., 2002). Because this cell line contains the SV40 large antigen, it is not suitable for functional studies on p53 which are expected to be bound and inactivated by the viral protein. To elucidate the pathway of p53-independent p21 is our next important task, further studies using truly p53-proficient cell lines are needed to clarify the role of p53.

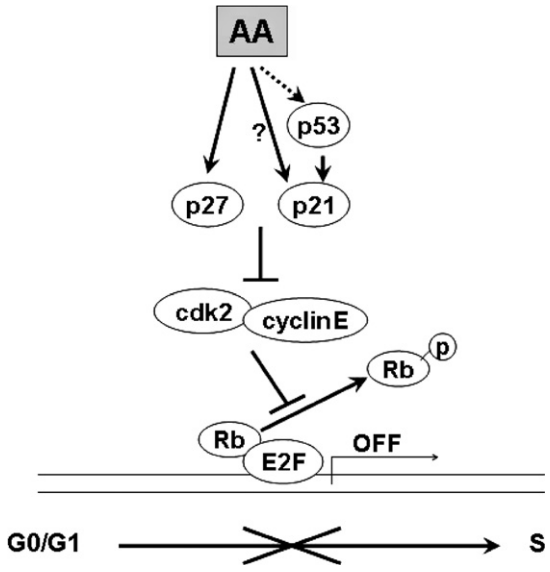

Fig. 5. A proposed model for the AAM-mediated cell cycle deregulation of SV-HUC-1 cells.

Based on our work, AAM causes the levels of p27, p21, p53 to remain elevated. A reduction in cyclin E/cdk2 complex leads to an increase in the free form of cdk2, thereby resulting in deficient phosphorylation of Rb, causing the cell cycle G0/G1 arrest.

Conflict of interest statement

None declared.

Acknowledgement

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