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Asparagus cochinchinensis inhibits the ethanol-induced cytotoxicity in Hep G2 cells

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Running title: *Asparagus cochinchinensis* in Hep G2 cells

SUMMARY

A human hepatoma cell line, Hep G2 cells are a reliable for the study of alcohol-induced hepatotoxicity. In this study, the author investigated the effect of an aqueous extract of *Asparagus cochinchinensis* $_{\text{MERRIL}}$ (Liliaceae) roots (ACAE) on ethanol (EtOH)-induced cytotoxicity in Hep G2 cells. ACAE dose-dependently inhibited the EtOH-induced tumor necrosis factor-α (TNF-α) secretion. ACAE also inhibited the EtOH and TNF-α-induced cytotoxicity. Furthermore, the author found that ACAE inhibited the TNF-α-induced apoptosis of Hep G2 cells. These results suggest that ACAE may prevent the EtOH-induced cytotoxicity through inhibition of the apoptosis of Hep G2 cells.

Keywords: *Asparagus cochinchinensis* $_{\text{MERRIL}}$ (Liliaceae) roots; Hep G2 cells; Ethanol; Tumor necrosis factor-α; Cytotoxicity; Apoptosis

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INTRODUCTION

Ethanol (EtOH) is almost exclusively metabolized by the liver (Lieber, 1985) and involves three main metabolic pathways localized in three different subcellular compartments of the liver cell (alcohol dehydrogenase and aldehyde dehydrogenase in the cytosol, the microsomal ethanol oxidizing system in the endoplasmic reticulum and the catalase in both peroxisome and mitochondria) (Teschke *et al.*, 1976; 1977).

Blood monocytes of patients with alcoholic liver disease generate amounts of tumor necrosis factor-α (TNF-α) and interleukin (IL)-1 and IL-6 at rates that are three to six times those for control values (McClain and Cohen, 1989; Deviere *et al.*, 1990). A good correlation between plasma IL-6 levels and mortality has been reported, and IL-6 levels normalized in parallel with clinical recovery (Khoruts *et al.*, 1991; Sheron *et al* ., 1991). Studies have focused on the involvement and mode of action of TNF- α and IL-1α in regulating hepatic acute phase reaction (Mackiewicz *et al.*, 1992; Healy and Gelehrter, 1994). By using human cell line able to regulate the synthesis of a broader spectrum of proteins *in vitro* (Baumann *et al.*, 1987; Kobsel and Ramadori, 1994) multiple factors likely to act on the liver cells *in vivo*

were studied (Jayaraman *et al.*, 1990; Greenwel *et al.*, 1995). The exposure of a human hepatoma cell line, Hep G2, to EtOH was followed by biochemical and morphological features in vitro comparable with the changes that may be observed in patients with alcohol-induced liver disease (Neuman *et al.*, 1993; 1995). Several investigators showed that this cell line displays drug metabolic functions (Dawson *et al.*, 1985; Marselos *et al.*, 1987; Cameron *et al.*, 1998).

Asparagus cochinchinensis MERRIL (Liliaceae) roots, a traditional Oriental medicine, has been used for treatment of various liver disease in Korea. The active principle, β-sitosterol, of this plant has been observed to have a effect of mouse S-180 leukemia and lung cancer (Huang, 1993). Recently Kim *et al.* reported that an aqueous extract of *Asparagus cochinchinensis* (ACAE) inhibited the secretion of TNF-α from primary cultures of mouse astrocytes stimulated with substance-P and lipopolysaccharide (Kim *et al.*, 1998).

In the present study, the author assessed the contribution of an ACAE in EtOH-induced cytotoxicity of Hep G2 cells. The author shows that ACAE inhibits the EtOH-induced cytotoxicity of Hep G2 cells.

MATERIALS AND METHODS

Reagents

EtOH was purchased from Alcohol Ltd. (Toronto, Ontafio, Canada). Bovine serum albumin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte razolium bromide (MTT), sodium EDTA, RNase and propidium iodide (PI) obtained from Sigma Chemical Co. (St. Louis, MO). α-Minimum essential medium (α-MEM), calcium chloride and fetal bovine serum (FBS) were purchased from GIBCO (Burlington, Ontario, Canada). Trypsin was obtained from Difco (Detroit, MI). Human recombinant TNF-α (rTNF-α) and TNF-α antibody obtained from R&D system Inc. (USA).

Cell cultures

A human hepatoma cell line, Hep G2 cells were obtained from the American Type Culture Collection (ATCC), Rockville, MD. Cells were seeded in 100-mm Falcon culture dishes or microplates (1×10^6 cells/ml; Falcon, Mississauga, Ontario, Canada). Hep G2 cells in long-term cultures were grown in α-MEM supplemented with 10% heat inactivated FBS 1% nonessential amino acids, 1% glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin. Cells were maintained in a humidified atmosphere of 95% O_2 - 5% CO_2 at 37℃; pH of the media was monitored at 7.4. Control cells were exposed for 24 h only to plain medium, or ACAE. Treated cells were exposed to 10, 80, and 100 μM/ml EtOH for 24 h. Hep G2 cells were also exposed for 24 h to 30 pg/ml rTNF-α in the presence or absence of ACAE.

Preparation of ACAE

The plant sample was obtained from the Oriental drug store, Bohwa Dang (Iksan, Korea). A voucher specimen (Number 4-97-41) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University. An ACAE was prepared by decocting the dried prescription of herbs with boiling distilled water. The duration of decoction was about 3 h. The decoction was filtered, lyophilized and kept at 4℃. The yield of dried extract from starting crude materials was about 8%.

MTT assay

The number of living cells in 96-well plates was determined by the MTT assay (Mosmann, 1983). MTT was dissolved in phosphate buffered saline (PBS) at a concentration of 5

mg/ml. From this stock solution, 10 μl per 100 μl of medium was added to each well, and plates were incubated at 37℃ for 2 h. Treated cells were then photographed. Treatment of living cells with MTT produces a dark blue formazan product, whereas no such staining is observed in dead cells. The numbers of MTT-positive (dark blue) and MTT-negative (white) cells were determined by counting the numbers of blue and white cells in four independent fields containing about 300 cells per field.

Assay of TNF-α secretion

TNF-α concentration in the cells derived culture supernatants was measured by a modified ELISA, as described (Kim *et al.*, 1999). The ELISA was devised by coating 96-well plates of human monoclonal antibody with specificity for human TNF-α. Before use and between subsequent steps in the assay, coated plates were washed with PBS containing 0.05% tween-20. All reagents used in this assay were incubated for 2 h at 37℃. Recombinant human TNF-α were diluted and used as a standard. Serial dilutions starting from 1 pg/ml were used to establish the standard curve. Assay plates were exposed sequentially to rabbit anti-TNF-α antibody, and phosphatase conjugated goat anti-rabbit IgG antibody and avidine peroxidase, and ρ-nitrophenyl phosphate and ABTS substrate solution containing 30% H_2O_2. The plates were read at 405 nm.

Analysis of apoptosis by PI staining

Proportions of cell cycle phase were analyzed by flow cytometry. In brief, the cells were fixed with 70% ethanol at 4℃ for 60 min. After washing with PBS, the cells were treated 0.5 ml of RNase and then with 1 ml of PI (100 μg/ml in PBS) solution in dark at 4℃ for 30 min. After washing and passing through nylon mesh, the samples were kept on ice until measured. The DNA histogram was obtained with a flow cytometry cell sorter (Becton Dickinson).

Statistical analysis.

Collected data were expressed as mean ± S.E. Statistical analysis was performed by the Student's t-test to express the difference between two groups.

RESULTS

The effect of the exposure to increasing EtOH concentration on the cell viability of Hep G2 cells, as assessed by the MTT test, is shown in Fig. 1.

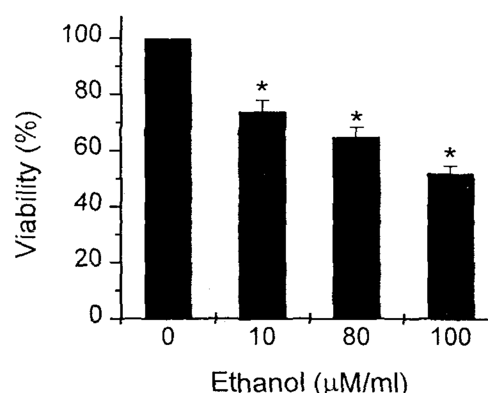

Fig 1. Effect of EtOH on cell viability and TNF-α secretion in Hep G2 cells. The cells (1×10^6 cells/ml) were incubated with α-MEM (control), 10, 80, and 100 μM/ml EtOH for 24 h. The cell viability was measured in three different plates in triplicate using MTT assay. *$P < 0.05$ *versus* medium alone control.

The cells were incubated with 0 (control), 10, 80, and 100 μM/ml EtOH for 24 h. EtOH were dose-dependently reduced the cell viability at doses of 10-100 μM/ml. In previous reports, EtOH has been reported to induce the TNF-α secretion (Neuman *et al.*,

1998). In order to confirm the EtOH-induced TNF-α secretion, Hep G2 cells were incubated with various concentrations of EtOH and then the secretion of TNF-α was measured by ELISA. EtOH were significantly increased the secretion of TNF-α (data not shown). To examine whether the production of TNF-α contribute to EtOH-induced cytotoxicity, Hep G2 cells were incubated with EtOH or human rTNF-α in the presence or absence of anti-TNF-α antibody. As shown in Fig. 2,

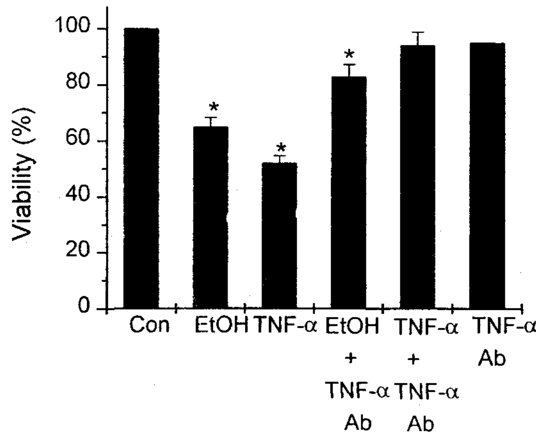

Fig 2. Effect of TNF- on cell viability in Hep G2 cells. The cells (1×10^6 cells/ml) were incubated with α-MEM (control), 80 μM/ml EtOH, 30 pg/ml Rtnf-α, 30 pg/ml anti-TNF-α antibody, or a combination of them for a period of 24 h. The cytoviability was measured in three different plates in triplicate using MTT assay. *$P < 0.05$ *versus* medium alone control

EtOH and rTNF-α was significantly reduced on the cell viability by itself, respectively. When EtOH was used in combination with the antibody against the TNF-α partially increased the cell viability. These results suggest that EtOH-induced cytotoxicity depend on the secretion of TNF-α in Hep G2 cells.

Previous work has shown that ACAE inhibited the secretion of TNF-α from astrocytes (Kim *et al.*, 1998). In this study, the author investigated the ability of ACAE to influence EtOH-induced TNF-α secretion in Hep G2 cells. TNF-α secretion was quantified by ELISA method. ACAE had no significant effect on TNF-α secretion by itself. However, EtOH (10 μM/ml) was markedly increased the secretion of TNF-α (Table 1). ACAE significantly inhibited the EtOH (10 μM/ml)-induced TNF-α secretion at doses of 1-100 μg/ml.

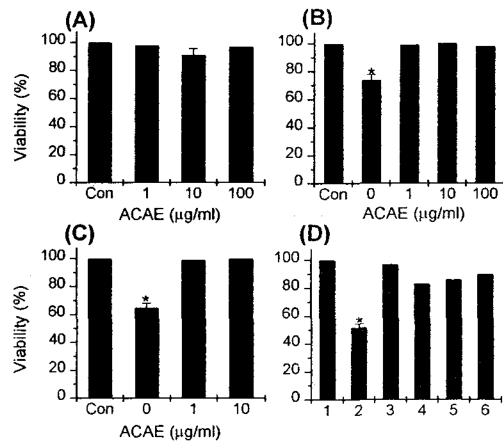

Fig 3. Effect of ACAE on EtOH- and TNF-induced cytotoxicity in Hep G2 cells. The cells (1×10^6 cells/ml) were incubated for 24 h in α-MEM (control) containing 10, 80 μM/ml EtOH and rTNF-α (30 pg/ml) with various concentrations of ACAE. Panel A (ACAE alone), panel B (10 μM/ml EtOH plus ACAE), panel C (80 μM/ml EtOH plus ACAE), and panel D (lane 1, control; lane 2, rTNF-α; lane 3, rTNF-α plus anti-TNF-α antibody; lane 4, rTNF-α plus 1 μg/ml ACAE; lane 5, rTNF-α plus 10 μg/ml ACAE; lane 6, rTNF-α plus 100 μg/ml ACAE). The cell viability was measured in three different plates in triplicate using MTT

assay. Each data value indicates the mean ± S.E. of five separated experiments. *$P < 0.05$ *versus* medium alone control.

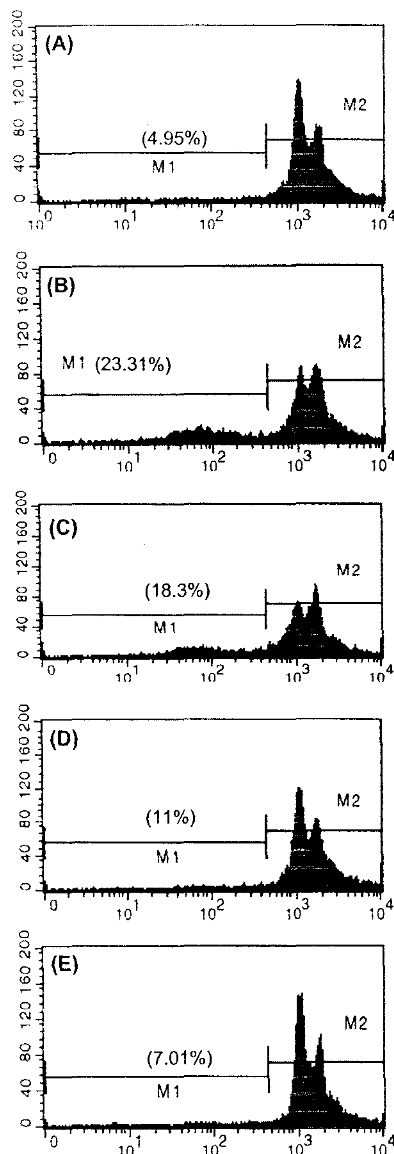

Fig 4. Effect of ACAE on the cell cycle distribution of Hep G2 cells. The cells were treated for 30 min with ACAE and then exposed to TNF-α at 37℃ for 24 h. Panel A, control; panel B, 30 pg/ml rTNF-α; panel C, 30 pg/ml rTNF-α + 1 μg/ml ACAE; panel D, 30 pg/ml rTNF-α + 10 μg/ml ACAE; and panel E, 30 pg/ml rTNF-α + 100 μg/ml ACAE. The cells were stained with PI solution and analyzed for DNA content by flow cytometry (see MATERIALS AND METHODS). Data represent the result from one of three similar experiments.

The author also investigated the ability of ACAE to influence EtOH-and TNF-α -induced cytotoxicity in Hep G2 cells. ACAE had no effect on the cell viability by itself (Fig. 3A). When cells pretreated with ACAE were exposed to 10 μM/ml and 80 μM/ml EtOH, EtOH-induced cytotoxicity markedly inhibit -ed compared with EtOH alone, respectively (Fig. 3B and 3C). In addition, to determine whether the inhibitory effect of ACAE in EtOH-induced cytotoxicity is also involved in the TNF-α-induced cytotoxicity in Hep G2 cells, cells pretreated with ACAE were exposed to 30 pg/ml of rTNF-α. As expected, cell viability of the cells exposed to TNF-α decreased significantly, from 100% control to 52%. TNF-α-induced cytotoxicity was completely blocked by treatment with the antibody against the TNF-α. When ACAE was used in combination with TNF-α dose-dependently inhibited the TNF-α-induced cytotoxicity at doses of 1-100 μg/ml (Fig. 3D). The effect of ACAE on TNF-α -induced apoptosis of Hep G2 cells, as determined by flow cytometry. As shown in Fig. 4, when cells were exposed to 30 pg/ml of rTNF-α for 24 h, apoptosis was increased by 23.31% compared with controls (4.95%). ACAE had no effect on apoptosis by itself (data not shown). When cells pretreated with ACAE were exposed to 30 pg/ml of rTNF-α, apoptosis was dose-dependently inhibited compared with the effect of rTNF-α alone.

Table 1. Effect of ACAE on TNF-α secretion by Hep G2 cells [a]

Treatment		TNF-α secretion (pg/ml)
EtOH (10 μM/ml)	ACAE (μg/ml)	
-	-	35±1.41
-	1	32±1.15
-	10	51±2.65
-	100	29±1.73
+	-	109±4.20
+	1	34±4.92*
+	10	42±2.64*
+	100	38±2.00*

[a] The cells (2 × 10^5 cells/well) were incubated for 24 h in medium alone or in medium containing EtOH (10 μM/ml) with various concentrations of ACAE. The supernatants were collected and frozen at −80℃ until assayed for TNF-α concentration. Each data value indicates the mean ± S.E. of three separated experiments. *$P < 0.05$ *versus* EtOH-treated control.

DISCUSSION

Hep G2 cell line is a reliable *in vitro* model for the study of alcohol-induced hepatotoxicity (Neuman *et al.*, 1995; 1998; Cameron et al., 1998). The damage is both time- and dose-dependent, and it is maximal when cells are exposed to EtOH for 24 h. In this study, the author have demonstrated that ACAE inhibited EtOH-induced cytotoxicity in Hep G2 cells. A number of studies have shown that levels of the TNF-α and IL-6 are elevated in the plasma of patients with alcoholic hepatitis (Felver *et al.*, 1990; Bird *et al.*, 1990). However, it is unclear of this elevation was associated with hepatic damage or, alternatively, was one of the causes of the toxic effect. As reported in Table 1, in vitro, exposure of Hep G2 cells to EtOH resulted in a marked increase in TNF-α. This fact suggests that EtOH induce release of TNF-α in Hep G2 cells. To define the role of TNF-α in producing the damage, human recombinant TNF-α was added to the cells and the cytotoxicity was measured thereafter (Fig. 2). This addition of TNF-α, alone or in combination, at concentrations similar to those observed after exposure to EtOH, did not result in an increased toxicity (data not shown). These results indicate TNF-α as a potential candidate in accounting for the EtOH induced toxicity measured in our in vitro model. This conclusion is further supported by the observation that incubation of Hep G2 cells with anti-TNF-α antibody may significantly reduce the cytotoxic effect of TNF-α.

Although low levels of TNF-α are actually required for cell proliferation (Akerman *et al.*, 1992), high levels are involved in experimentally induced hepato-toxicity (Ostenson *et al.*, 1987; Coletti et al., 1990; Naume *et al.*, 1991). Addition of anti-TNF-α antibody in our system, however,

was partially inhibited the EtOH-induced toxicity. This indicates that TNF-α is partially responsible for the cytotoxic effect of EtOH and metabolic alterations should be also considered.

In summary, ACAE dose-dependently inhibited the EtOH-induced cytotoxicity and secretion of TNF-α. Moreover, ACAE inhibited the TNF-α-induced apoptosis in Hep G2 cells. The author concluded that EtOH-induced cytotoxicity is a complex process in which other mechanisms in addition to cytokines are involved. In addition, the possibility that ACAE possesses the inhibitory effect of IL-1 and IL-6 secretion and that the effect might contribute to the EtOH-induced cytotoxicity of ACAE cannot be excluded. Although caution should be taken in extrapolating data obtained in an *in vitro* model to *in vivo* conditions, ACAE-induced down regulation of TNF-α may have a beneficial role in the treatment of alcohol-induced liver damage.

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